Intracellular bacteria as DNA carriers *in vitro* and *in vivo*

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1 Introduction

1.1 Gene Therapy

Conventional treatment of monogenic diseases like Cystic Fibrosis aims mainly at relieving the symptoms rather than addressing the cause of the disease. Somatic gene therapy in contrast could be used to complement a defective gene with a functioning one in order to cure the disease, slow its progression, or even prevent its onset. Other approaches in gene therapy involve the treatment of complex diseases like cancer. Here, the cause of the disease is addressed for example by transferring genes that interact with cell proliferation or genes that encode for immune stimulatory factors.

In both cases, the basic principle relies on the transfer of genetic material (genes or cDNA) to host cells that are either directly involved in the disease or can effect its course. Transfer can be achieved by *in vivo* or *ex vivo* methods. *In vivo* gene therapy implies the transfer of genetic material to cells by suitable vectors directly inside the organism. Using the *ex vivo* approach, cells – e.g. haematopoietic stem cells – are taken out of the body, the expression cassette for the therapeutic transgene is introduced *in vitro*, and the recombinant cells are transferred back into the host. Both methods have advantages and disadvantages. The *ex vivo* approach offers more efficient gene transfer and the possibility to control and select successfully manipulated cells. It is, however, patient specific due to the requirement of histocompatible cells, which renders the process more costly. *In vivo* gene transfer, in contrast, is less expensive, easier to handle and not patient specific, but also less efficient.

Essential for all gene therapy approaches are efficient vector systems. Such vectors can grossly be divided into viral and non-viral vectors. So far, viral vectors are superior to non-viral vectors in terms of transfection efficiency, transgene expression levels and duration of expression. This is also represented by the fact that 70% of clinical protocols involve viral gene delivery vectors (Fig. 1.1). Of these, 53% employ retroviruses or adenoviruses (discussed below).

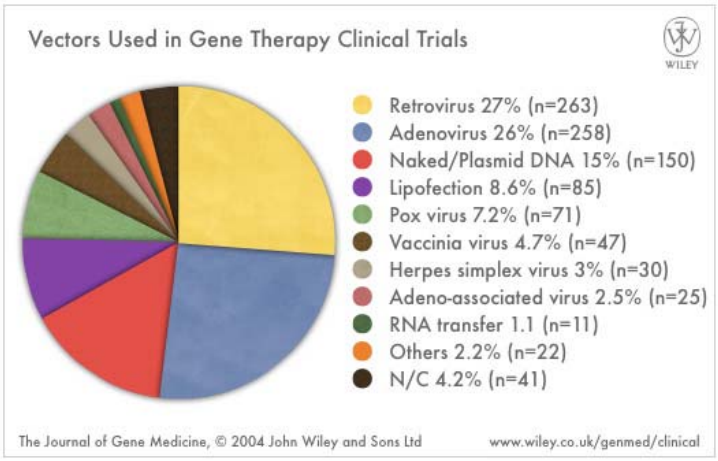

Fig. 1.1: Frequency of use of different vectors in gene therapy clinical trials.

Non-viral vectors comprise mainly naked DNA (notably used in 15% of gene therapy clinical trials), or DNA complexed with cationic liposomes or polymers. Recently, also intracellular bacteria have emerged as potential gene delivery systems.
The criteria for an ideal gene therapy vector are reviewed below (from [157]).

Easy production

The vector should be easy to produce at high titre on a commercial scale. This consideration stems from the wide range of cell numbers that must be transduced — from a handful of stem cells capable of reconstituting the entire haematopoietic repertoire to 10^{11} or more cells to infect 5–10% of hepatocytes in the liver. For widespread use, the vector should be amenable to commercial production and processing (such as concentration technology for delivery in small volumes), and should have a reasonable shelf-life for transport and distribution.

Sustained production

The vector, once delivered, should be able to express its genetic cargo over a sustained period or expression should be regulable in a precise way. Different disease states have different requirements (for example, regulated expression in diabetes and lifetime expression in haemophilia).

Immunologically inert

The vector components should not elicit an immune response after delivery. A humoral antibody response will make a second administration of the vector ineffective, whereas a cellular response will eliminate the transduced cells.

Tissue targeting

Delivery to only certain cell types is highly desirable, especially where the target cells are dispersed throughout the body (such as in the haematopoietic system), or if the cells are part of a heterogeneous population (such as in the brain). It is also important to avoid certain cells, such as dendritic cells, the "professional" antigen-presenting cells of the body, because of their role in mediating the immune response. Cell or tissue-targeted vectors present a great challenge, but also offer rich dividends for gene therapy approaches.

Size capacity

The vector should have no size limit to the genetic material it can deliver. The coding sequence of a therapeutic gene can vary from 350 base pairs for insulin, to over 12,000 base pairs for dystrophin. Furthermore, addition of appropriate regulatory sequences may be required for efficient transduction and expression of the foreign genetic material.

Replication, segregation or integration

The vector should allow for site-specific integration of the gene into the chromosome of the target cell, or should reside in the nucleus as an episome that will faithfully divide and segregate on cell division. Site-specific integration is a very desirable attribute because it eliminates the uncertainty of random integration into the host chromosome, and endogenous regulatory regions will control its expression under physiological conditions. The ability of the vector to be maintained as an episome could make the genetic elements independent of local chromatin environments, but faithful replication and segregation is needed if the vector is to be effective in systems such as stem cells.

Infection of dividing and non-dividing cells

As large numbers of cells (such as neurons, hepatocytes and myocytes) are postmitotic, vectors capable of efficiently transducing non-dividing cells are very desirable.

So far, no vector is available that matches all the criteria. Rather, different vectors are designed to fit special requirements.

1.2 Viral vectors

Viral vectors are derived from modified viruses in which genes essential for propagation are replaced by the transgene. The essential genes can be provided in *trans* by a so called packaging or helper cell, leading to the generation of viral particles that are infectious and can introduce their genetic material into a recipient cell. However, due to the lack of the essential genes, it cannot replicate any further. Viral vectors can be divided into two categories: integrating and non-integrating. The group of integrating vectors mainly comprises retroviral, lentiviral and adeno-

associated viral vectors [157]. These are especially suitable for life-long expression of the transgene, as required for complementation of genetic defects like Cystic Fibrosis or diabetes. Adenoviral vectors, in contrast, are maintained in the recipient cell as an episome.

Retroviral vectors

Retroviruses are positive-sense single-strand RNA viruses. After introduction of their genetic material into the host cell, reverse transcription of the viral genome into double-stranded DNA is initiated. Subsequently, the DNA is integrated into the genome of the host cell and the viral proteins needed for propagation are expressed. Retroviral vectors were among the first gene therapy vectors to be designed.

Usually, retroviral vectors consist only of long terminal repeats (LTRs) and a packaging function but lack the *gag* (encoding viral structural proteins), *pol* (encoding reverse transcriptase/ integrase) and *env* (encoding viral envelope glycoprotein) genes. These essential proteins are provided by a packaging cell in *trans* during the manufacture of the retroviral vector. Separating *gag* and *pol* from *env* in the packaging cell significantly reduced the risk of generating replication-competent virus by recombination of the different gene segments. Meanwhile, a wide variety of packaging cell lines and vectors are available, equipped with particular features such as tissue specific or inducible promoters and env proteins with modified target specificity. However, retroviral vectors have one major restriction: they can transduce only dividing cells because the linear DNA that is synthesized during the viral infection is unable to penetrate the intact nuclear membrane. Hence, organs like brain, lung, pancreas or the eye are not accessible to *in vivo* gene transfer. Nevertheless, retroviral vectors are still extensively used to transduce dividing cells such as tumour cells or haematopoietic cells [157].

Lentiviruses like HIV belong to the family of retroviruses, but have the unusual property to transduce dividing as well as non-dividing cells. Consequently, systems based on lentiviruses have been developed. In the first generation, the env protein was substituted by vesicular stomatitis virus G protein (VSVG), relieving these vectors of their dependence on CD4. However, concerns were raised because of the possibility of generation of infectious HIV by recombination. To address this issue, many viral accessory genes were deleted, such that the latest lentiviral vector

systems retain less than 25% of the viral genome in the packaging construct and less than 5% of the viral genome in the vector construct [76; 189]. The improved vectors are able to transduce a wide variety of host cells. Data from clinical trials using lentiviral vectors are currently not available and the duration of transgene expression from these vectors also needs further testing.

Adenoviral vectors

Adenoviruses are double-stranded DNA viruses that cause respiratory tract infection in humans. These viruses exhibit a broad host range and cell tropism. Replication-defective adenoviral vectors have been generated by deleting several viral genes, and high titre preparations of these recombinant vectors can be produced with relatively little effort.

A big advantage of adenoviral vectors over retroviral vectors is their ability to transduce dividing as well as non-dividing cells. In the host cell, the adenoviral genome is maintained as an episome in the nucleus. Therefore the major obstacle of adenoviral vectors for general use concerns the persistence of transgene expression. With the first generation of adenoviral vectors, sustained transgene expression was observed only in long lived cells (such as muscle cells and neurons) in immuno-compromised animals, while in immuno-competent individuals an immune response was raised against the recombinant adenoviral vector. Cellular immune responses eliminated the transduced cells, while humoral immunity prevented repeated administration of the vector [26; 77]. For these reasons, adenoviral vectors are mainly used for applications that require high level but transient transgene expression, like anti-cancer therapy.

In the more advanced form, the so called “gutless” adenoviral vectors, the viral protein encoding sequences have been deleted and are provided in *trans* by a helper virus [83; 121]. With these vectors, the above mentioned problems appear to be solved, and long term maintenance of the transgene has been observed in several organs and different species (reviewed in [64; 84]).

1.3 Non-viral vectors

The major advantages of non-viral vectors are their simplicity of use, ease of large scale production and lack of initiation of a specific immune response against the vector [95]. Safety concerns about the use of viral vectors in humans render these delivery systems a serious alternative. The major limitations of the non-viral vectors are their relatively low transfection efficiency as compared to viral vectors and the short duration of transgene expression. To achieve more efficient gene transfer, the plasmid DNA was complexed together with cationic liposomes or polymers. This has improved transfer efficiency *in vitro*. *In vivo*, however, the effect is strongly dependent on the route of administration. Some efforts have been made to compensate for these restrictions, including the development of episomal vectors for prolonged transgene expression, and cytoplasmic expression vectors to circumvent the necessity of nuclear transport of the DNA, thereby enhancing the number of cells expressing the gene of interest.

An interesting aspect of direct gene transfer was discovered by Wolff and co-workers in 1990. They described direct gene transfer after intramuscular injection of plasmid DNA into mice [184]. This elicited an efficient immune response against the antigen encoded by the vector. Subsequently, this approach has been used to express a wide range of antigens, in particular viral and tumour antigens, thereby protecting mice against various infectious agents and tumour challenges. This phenomenon has been named “DNA vaccination”. The efficiency of gene transfer via naked DNA was further improved by alternative physical application methods, such as electroporation and the use of a gene gun.

1.4 Bacteria-mediated gene transfer to mammalian cells

As mentioned before, vector systems used so far suffer from low transfection efficiencies *in vivo* and/ or sometimes even raise safety concerns. Recently, intracellular bacteria emerged as a new class of vectors for gene delivery to mammalian cells and might be established as serious alternative to the commonly used systems (reviewed in [97]). The first direct transfer of plasmid DNA from *Escherichia coli* to a mammalian cell was described already in 1980 by Schaffner [142]. In the meantime, several invasive bacteria have been used for gene transfer

approaches, including attenuated strains of the Gram-negative species *Salmonella typhimurium* and *Shigella flexneri*, the Gram-positive bacterium *Listeria monocytogenes* as well as engineered invasive *Escherichia coli,* to name the species studied most extensively. It should be mentioned here that also non-invasive bacteria like *Agrobacterium tumefaciens* are able to directly transfer DNA to eukaryotic host cells by using their conjugation apparatus [90].

In all protocols in which invasive bacteria are used to date, the transfer of genetic material to the host cell is dependent on the death and lysis of the bacterium inside the cell. This can be achieved by different methods. In the first studies, auxotrophic mutants of *S. flexneri* with a disrupted *asd* gene encoding an essential component for the synthesis of the bacterial cell wall were used [22; 153]. Other protocols involved bacteriophage lysis components or, as in our own protocol, antibiotics were used to induce bacterial death [29; 60]. Spontaneous transfer of plasmid DNA from wild type bacteria has been reported as well [55; 88]. In that case, death of the bacteria might have been induced by host cell defence mechanisms or temperent phages.

The mechanism of DNA transfer from different bacteria is shown schematically in Fig. 1.2. Escape from the phagocytic vacuole to the cytoplasm of the host cell by production of membrane disrupting toxins is a characteristic shared by Listeria and Shigella. Once in the cytoplasm, the lysis of the bacteria is induced and the plasmid load can be released. In the case of Listeria, escape from the vacuole was shown to be essential for gene transfer to take place [60]. Salmonella and some of the *E. coli* strains used, however, do not share this feature [97]. After invasion of the cell, these bacteria remain in the vacuole. Nevertheless, they mediate transfection of host cells after induction of bacterial lysis. How the transfer of plasmid DNA in this case is occuring is not yet understood. Also, the mechanism by which the plasmid DNA eventually reaches the nucleus is not clear.

Different bacteria exhibit different host cell specificities *in vitro* with regard to their ability to mediate gene transfer. Salmonella, for example, transfect *in vitro* only primary murine and human macrophages and to some extend dendritic cells [15; 27; 49; 55]. This specificity renders them especially suitable for genetic vaccination. In contrast, *L. monocytogenes* and invasive *E. coli* are able to transfect various cell lines mainly of epithelial origin, but have also been shown to act on primary cells [55]. Salmonella-mediated transfection of cells other than macrophages can be achieved by conferring constitutive secretion of Listeriolysin O (LLO), the pore forming toxin of

L. monocytogenes, to the bacteria. This allowed the recombinant Salmonella to escape from the phagosome, similar to Listeria [49].

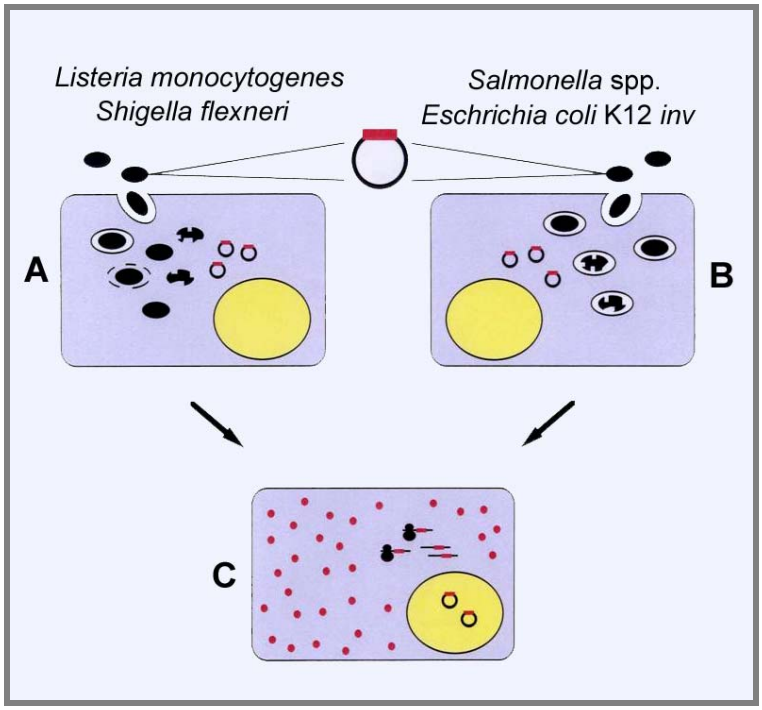

Fig. 1.2: Principles of bacteria-mediated gene transfer to eukaryotic host cells (from [183]). A) Some intracellular bacteria like *L. monocytogenes* or *S. flexneri* can escape from the phagosome after entry of the host cell. They lyse and release their plasmid DNA into the cytosol. B) Other bacteria like Salmonella or invasive *E. coli* are unable to escape from the phagosome and are lysed within this vesicle. How the plasmid DNA is transported to the cytosol is unknown. C) In both cases, the plasmid reaches the nucleus where the transgene is transcribed and later, in the cytosol, translated into the protein of interest.

Bacteria as vectors for gene transfer have several advantages, including the different host tropisms mentioned above. Many bacteria (as well as the plasmid DNA they carry) are genetically easy to manipulate. Furthermore, the bacterial carrier and the plasmid to be transferred are distinct entities that can be modified independently, allowing the combination of different properties. The production and handling of bacterial vectors is simple and inexpensive. Most importantly, bacteria like Listeria or Shigella, that can spread from cell to cell, might be able to reach deeper tissue layers *in vivo* which are not accessible to immobile synthetic or replication incompetent viral vectors.

So far, the main focus in the field of bacteria-mediated gene transfer has been directed to genetic vaccination. In this approach, the genetic information for an antigen of a particular pathogen is delivered to the host, where it is expressed and a specific immune response is triggered. Bacteria as carriers for this genetic material confer ease of application, the ability to target immune inductive organs and cells and possess powerful adjuvant properties [97]. Most experience was collected by the use of different recombinant attenuated Salmonella strains, in animal models, in primates as well as clinical trials [123; 124; 182]. Such strains have been successfully used as carriers for DNA vaccines in immunization protocols against a variety of pathogens like bacteria, viruses, fungi and parasites, but also for protection or therapy of cancer (reviewed in [97]).

Salmonella have not only been used for immunization, but also for gene therapy applications, e.g. to complement in vitro the monogenic defect in macrophages from patients with hereditary haemochromatosis by transferring the HFE gene [110]. Application of attenuated Salmonella carrying an expression plasmid for interferon γ (IFN-γ) to IFN-γ deficient mice rescued the mice from a lethal challenge with Salmonella [118]. Other examples for *in vivo* gene therapy mediated by Salmonella include transfer of CD40L, which protected the mice from a B cell lymphoma challenge [173], and transfer of the genes for interleukin-12 (IL-12) or granulocyte-macrophage colony-stimulating factor (GM-CSF), both leading to the expression of the particular cytokine and suppression of tumour growth [187].

Recent developments in bacteria-mediated gene transfer involve the use of bacterial artificial chromosomes (BACs), as was shown by transfer of such large DNA fragments to HeLa cells by an invasive *E. coli* strain *in vitro* [113]. This system has virtually no size limitation for the transferred genetic material. Fragments of up to 1 Mb can be carried. Furthermore, transfer of double stranded RNA from *E. coli* to various cells of the nematode *C. elegans* was shown, leading to target specific gene silencing by RNA interference [168; 169]. These findings impressively underline the potential of bacteria as carriers for therapeutic agents in a wide variety of applications, including vaccination against infectious diseases, complementation of monogenic defects and cancer gene therapy.

1.5 Cancer Gene Therapy

Of all clinical gene therapy trials, 66% are addressing cancer (http://www.wiley.co.uk/genmed/clinical). Cancerous cells exhibit properties that distinguish them from normal, healthy cells of the body. These differences can be used to specifically direct treatment to abnormal cancer cells while leaving normal cells unaffected. Thereby adverse side effects are minimized.

Development of tumours is associated with multiple alterations on the genetic level. Uncontrolled growth is supported for example due to mutations that result in over-expression of proto-oncogenes or down-regulation of tumour-suppressor genes [39]. As long as these two gene groups are in balance with each other, cell proliferation and death is properly controlled. Disturbance of this balance leads to uncontrolled cell proliferation. More mutations are acquired during this process and the transformed cells become more and more malignant and heterogeneous with respect to each other. Therefore, effective treatment should address aspects that are common to all cancers. Several such strategies have been developed recently, including oncogene inactivation, tumour suppressor gene replacement, inhibition of angiogenesis, immunopotentiation and molecular chemotherapy [62]. Different systems have been used to realize these strategies.

Oncogene inactivation: Targeted disruption of proto-oncogenes is one promising strategy for cancer gene therapy. These genes are important during fetal development to achieve sufficient amounts of cells in all organs, but most of them are silenced afterwards to prevent abnormal tissue growth. Inactivation of these genes can be achieved on different levels of gene expression: (1) inhibition of oncogene transcription into mRNA; (2) reduction of mRNA translation into protein; (3) interference with oncoprotein transportation and function [62]. Transcription of genes can be, for example, blocked by using DNA oligonucleotides that bind specifically to the promoter region of the gene of interest, forming triple helix structures that prevent the binding of the RNA polymerase complex and subsequently transcription into mRNA [35; 80-82; 92].

A similar approach to reduce oncogene translation relies on the use of oligonucleotides that bind to mRNA in a complementary manner. The resulting double-stranded mRNA molecules are then degraded by intracellular enzymes. This approach has been used (in combination with chemotherapy) to block bcl-2

oncogene translation in prostate and breast cancer cells [99; 170]. However, the ability of these oligonucleotides to reduce translation is concentration dependent and therefore tumour cells often continue to produce low levels of oncogenes.
Most recently, so called ribozymes have been developed, i.e. specialized RNA molecules that bind and cleave targeted mRNA sequences, thus preventing translation into protein. This technique has been employed to destroy over-expressed mRNAs in a variety of cancers [1; 8; 75; 156; 162; 167].
To interfere with oncogenes on the protein level, intracellular single chain antibodies ("intrabodies") have been used. After transfer of the coding sequence into the cancer cell, the intrabodies are expressed and bind to specific oncogenes, hence preventing the interaction of the oncoprotein with its target. Intrabodies have been developed against p53, erb2 and cyclin E proteins that are over-expressed in ovarian and breast cancers [3; 17; 160].

Replacement of tumour suppressor genes: the proteins encoded by tumour suppressor genes generally are involved in the cell cycle control and mediate DNA repair functions. Damage of DNA usually triggers cell cycle arrest and induces DNA repair mechanisms. If the damage cannot be corrected, the cell undergoes apoptosis. In case the expression of tumour suppressor genes is inhibited, cells with altered DNA sequences can continue to proliferate and propagate their mutations to daughter cells. Since DNA repair is not triggered by the defective tumour suppressor genes, the cells will acquire new mutations and at the same time avoid apoptosis. As consequence, they will become dedifferentiated with time. Several tumour suppressor genes have been shown to arrest growth and induce apoptosis when transfected into cancer cells, including p53, Rb and PTEN [11; 43; 45; 108; 145; 151].

Inhibition of angiogenesis: one feature that is common to all solid tumours regardless of their origin is their dependence on adequate blood supply that meets the demands for nutrients and oxygen. As a logical consequence, gene therapies have been developed that interfere with angiogenesis and tumour adaptation to hypoxia. Gene transfer of angiostatin and endostatin, two potent natural inhibitors of angiogenesis, has led to decreased angiogenesis and reduced tumour growth in different models [16; 149; 161]. Anti-sense oligonucleotides against a subunit of $\alpha v\beta 3$ integrin diminished angiogenesis in transfected human endothelial cells [87]. The same technique was used to target hypoxia inducible factor-1 (HIF-1), a transcription

factor that is up-regulated by low oxygen concentrations and activates angiogenesis-associated genes (e.g. vascular endothelial growth factor, VEGF). Clinically, over-expression of the alpha subunit of HIF-1 correlates with treatment failure and mortality in a variety of human cancers. When mice were treated by antisense oligonucleotide gene therapy to HIF-1 alpha, down-regulation of VEGF, decreased microvessel density in tumours and rejection of small lymphomas were observed [161]. These results indicate that inhibition of angiogenesis might be a powerful tool in the therapy of solid cancers in general.

Immunopotentation: one of the reasons why cancer cells can spread in the body without being attacked by the immune system is their escape from immune recognition, e.g. by downregulating MHC molecules. In this case, altered tumour associated proteins cannot be presented to cytotoxic or helper T cells.
Immunopotentiation is the enhancement of the immune system's ability to destroy cancer cells. One can distinguish between passive immunopotentiation, which involves boosting of the natural immune response, and active immunopotentiation, which requires the initiation of an immune response against a previously unrecognized tumour. Strategies for passive immunopotentiation include transfection of cytotoxic T cells, NK cells, macrophages and dendritic cells with genes encoding cytokines or growth factors *ex vivo* [125]. Also, immune stimulatory cells can be pulsed with RNA from tumour cells in order to enhance an immune response against specific antigens of the tumour [66]. Strategies for active immunotherapy include genetic modification of tumour cells, e.g. by introduction of genes encoding MHC molecules, the co-stimulatory molecule B7, or cytokines. These cells are then irradiated and reintroduced into the patient [152].

Molecular chemotherapy: the principle of molecular chemotherapy or suicide gene therapy is based on the systemic administration of non-toxic pro-drugs that are turned into cytotoxic compounds only in previously transfected cancer cells. Into these cells genes are introduced that code for enzymes capable of converting the pro-drug into the active cytotoxic drug. Not only the directly modified cell is affected, since the toxin can be exported to neighbouring cells through gap junctions as soon as a certain level of the toxic compound is reached. Once the toxin reaches high enough levels, the originally transfected cell as well as the neighbouring cells die (bystander effect). Therefore, only a fraction of the cells in a solid tumour has to be transfected for successful suicide gene therapy.

A recent phase I study has shown that suicide gene therapy can be successfully and safely be used in humans. The authors used an adenoviral vector encoding both thymidine kinase of herpes simplex virus (HSV-TK) and cytosine deaminase (CD) from *E. coli* for therapy of locally recurrent prostate cancer [42].

These results show that intensive research is carried out in the field of cancer gene therapy. All the strategies presented above heavily rely on the efficient transfer of genetic material to tumour or effector cells. Mostly, viral vectors are used for this kind of therapy, since they confer the most effective transfection. However, due to recent events [56] viral vectors are met with significant concerns regarding safety in humans. Therefore a variety of non-viral vectors have been tested. These suffer yet from too low transfection rates *in vitro* and especially *in vivo*. Thus, the ideal vector for cancer gene therapy still has to be developed.
Intracellular bacteria have recently been shown to specifically target to and replicate in solid tumours [186]. This represents an exciting new possibility. Thus bacteria await to be explored in detail as vectors for targeted delivery of therapeutic genes to tumours.

1.6 Bacteria in cancer treatment

Several decades ago it was reported that some bacteria can take advantage of the growth conditions found in necrotic areas of solid tumours. Low oxygen concentration and high nutrient content led to exclusive or at least preferential proliferation of the microorganisms in tumour tissue. In the study of Malmgren and Flanigan [102], spores of the Gram-positive, obligate anaerobe bacterium *Clostridium tetani* were injected intravenously into tumour bearing mice. Although the spores were found throughout the body, only those that had entered the hypoxic regions of the tumour germinated and proliferated. Because of the particular pathogenic bacterium used, mice succumbed to tetanus poisoning 48 hrs after the infection, while control mice without a tumour, but infected with the same dose of spores, showed no signs of poisoning. Thereafter, several experiments involving different species of Clostridium were undertaken in mice and humans which led to the conclusion that growth of the microorganism in the tumour induces destruction of large parts of the malignant tissue (“oncolysis”). However, a viable, well oxygenated rim remained from which the tumour could re-establish itself. Nevertheless, these findings brought about the idea

to use bacteria as a vehicle to deliver anti-cancer agents selectively to tumour tissues.

The main focus so far was on the delivery of pro-drug converting enzymes, either by using engineered strains producing the protein itself or by using bacteria carrying a eukaryotic expression plasmid encoding the enzyme. The latter approach requires plasmid transfer from the bacteria to the tumour cell. Thus a clone of *Clostridium sporogenes* was used that carried a plasmid with the *Escherichia coli codA* gene [96]. The *codA* gene codes for cytosine deaminase, an enzyme that converts the non-toxic pro-drug 5-fluorocytosine (5-FC) into the cytotoxic 5-fluorouracil (5-FU). Spores of the recombinant strain were used to colonize tumours in C3H/Km mice, and the effect of systemic administration of 5-FC was addressed. Significant anti-tumour effects were observed that were at least equivalent to those observed after administration of the highest tolerated dose of the highly toxic chemotherapeutic 5-FU.

Other bacteria have also been reported to preferentially replicate in necrotic areas of solid tumours. When administered intraperitoneally into melanoma bearing C57/B6 mice, an attenuated auxotrophic strain of *Salmonella typhimurium* showed an up to 9000:1 ratio of the number of bacteria found in tumours to that found in liver. Tumour growth was suppressed and survival of the mice was prolonged already after treatment with the bacteria alone [126]. In addition, this strain was engineered to produce thymidine kinase of herpes simplex virus (HSV-TK). This enzyme phosphorylates the pro-drug ganciclovir (GCV). The resulting monophosphate form of GCV is subsequently converted by cytosolic enzymes into the triphosphate form, which intercalates into replicating DNA and leads to cell death. Application of this recombinant attenuated Salmonella strain and administration of GCV led to an even more pronounced tumour regression and survival of treated mice.

One problem of administering high doses of Gram-negative bacteria is their potential to induce a TNF-α mediated toxic shock, which is mediated by Lipid A, a compound of the bacterial cell wall. The *msbB* gene of *E. coli* and Salmonella is involved in the synthesis of this lipid. The disruption of this gene in Salmonella reduced TNF-α induction and increased the LD_{50} of this bacterium 10.000-fold. Nevertheless, the capacity to target into the tumour and suppress its growth was retained [100].

Lately, a similar phenomenon was described for attenuated strains of *Vibrio cholerea*, an extracellular bacterium, and *Listeria monocytogenes*, as well as the laboratory

strain *E. coli* DH5α. Yu et al. showed that these bacteria accumulated in tumours of nude mice after intravenous injection [186]. However, these mice lack functional T cells and therefore are immuno-compromised. The *V. cholerea* strain was able to colonize tumours also in immunocompetent C57/B6 mice and Lewis rats [186].
Alternative explanations for the selective accumulation of bacteria in tumours except special nutrients and low oxygen content in the tumour microenvironment have been proposed. One hypothesis is based on the finding that some bacteria tend to adhere to tumour cells but not to benign cells [34]. Another idea is that the impaired lymphatic system in tumours leads to reduced immunosurveillance, thereby protecting the bacteria from the immune system of the host [115].

1.7 Bacteria as transfer vehicles for macromolecules

Many bacteria species have been used as carriers for macromolecules into mammalian hosts. As mentioned above, these macromolecules comprise DNA [97], but also RNA [143; 168] and proteins [46; 48]. For vaccination purposes, heterologous expression of protein antigens in Salmonella, Shigella and Listeria has been shown to raise strong immune responses in the mouse. However, no success of this approach has been reported in humans. Regarding DNA transfer, Salmonella and Shigella have been mainly used for genetic vaccination, while Listeria were predominantly used for the transfer of complementing genes. In the present work, the potential of bacteria-mediated gene transfer for tumour therapy should be tested. Because of their broad host cell tropism and their ability to spread from cell to cell, Listeria and Shigella were employed for this purpose. Thus, these two bacterial carriers are described in more detail below.

1.7.1 *Listeria monocytogenes*

Listeria monocytogenes is a rod shaped Gram-positive, facultative anaerobic intracellular bacterium. It is motile at temperatures between 10 and 25°C, does not form spores and has no capsule. It is almost ubiquitously found in the environment, e.g. in soil, water, a large number of foods and faeces of humans and animals. *L. monocytogenes* can infect humans and a wide variety of other vertebrates. The disease caused by this bacterium is known as listeriosis, whose characteristic

features are sepsis, meningitis and encephalitis [40; 53; 144]. In healthy individuals, *L. monocytogenes* shows little virulence and infections are rapidly cleared by the host's immune system. If an infection can be established in a healthy person, it does in most cases not cause severe illness, but might be accompanied with flu-like symptoms and diarrhoea [53]. However, in newborns, elderly people or immune suppressed patients, the infection can be life threatening. Pregnant women are especially at risk, because the bacterium can cross the placental barrier [176] and infect the foetus, which in turn can lead to abortion, still birth or neonatal death. The microorganism is often taken up by contaminated food, like cheese and other milk products or smoked fish. It is able to survive high salt content and low pH values, and can proliferate at refrigerating temperatures, thereby representing a major problem in the safety of food processing.

Intracellular life cycle

Listeria monocytogenes is an invasive pathogen that can, besides phagocytic cells, enter several non-phagocytic cell types. The key features of its intracellular life cycle are summarized in Fig. 1.3. The cycle starts with the adhesion of the bacterium to the membrane of the eukaryotic host cell. The main bacterial factors involved in this step are internalin A (InlA) and internalin B (InlB) [21; 67]. InlA binds specifically to E-cadherin, with a stringency that was unexpected. While InlA is effectively interacting with human E-cadherin, it cannot interact with the mouse homolog of this protein, a difference that is due to a single amino acid exchange at position 16 of the polypeptide [93].

InlB, in contrast, has no such restricted host cell tropism and allows entry into a wide variety of cells [13; 67; 119]. gC1qR was identified as one of the receptors for InlB [12]. It is a surface protein that does not exhibit any features of a transmembrane or GPI-anchored protein, suggesting that this protein is interacting with another molecule to mediate the signals necessary for the rearrangement of the cytoskeleton involved in entry. Another receptor for InlB is the Met receptor tyrosine kinase, the receptor for hepatocyte growth factor. Interaction of InlB and Met leads to tyrosine phosphorylation of Met and the adapters Gab1 and Cbl, leading to *L. monocytogenes* invasion [148].

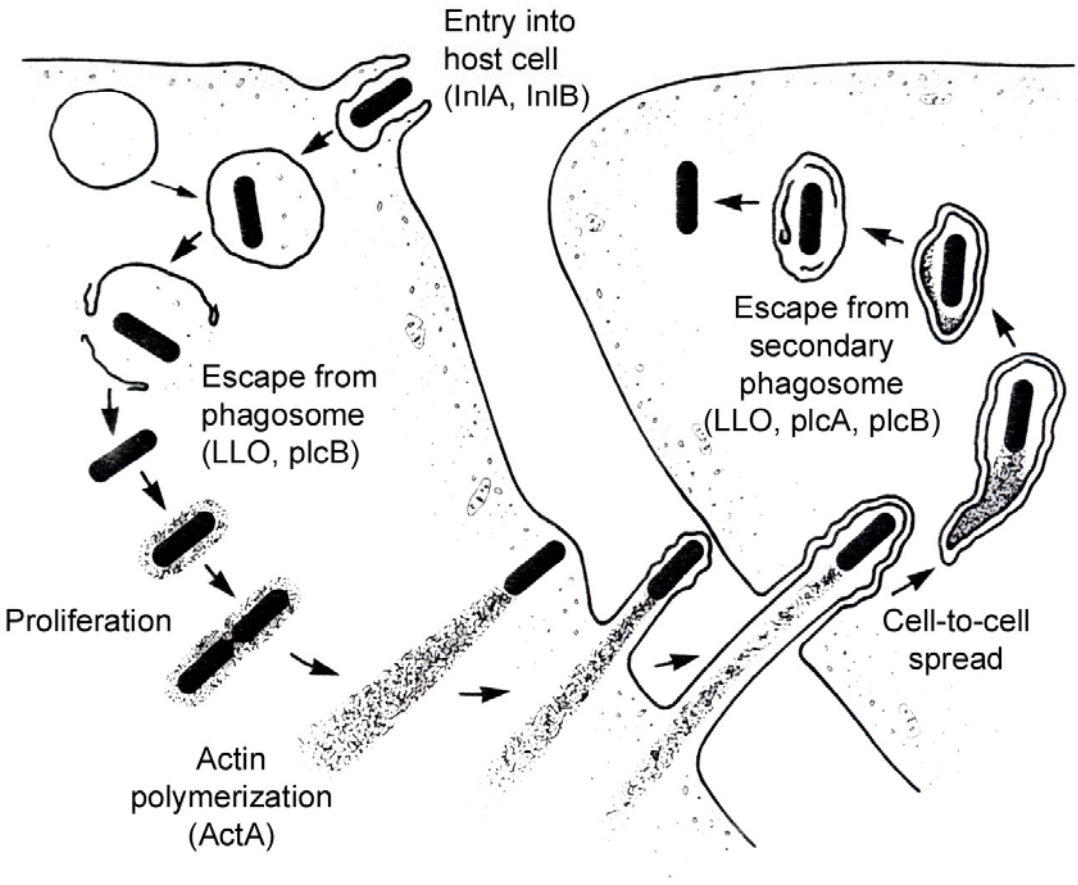

Fig. 1.3: Schematic view of the intracellular life cycle of *L. monocytogenes.* The different steps and the virulence factors involved are shown. See text for detailed explanation.

This interaction with the host cell membrane leads to rearrangements of the actin cytoskeleton and engulfment of the bacterium by a zipper-like mechanism. Subsequently, the bacterium is taken up into the cell in a phagocytic vacuole. The membrane of this vacuole is disrupted shortly after uptake by Listeriolysin O (LLO), in synergy with phospholipase C B (plcB). Strains expressing LLO variants that display less lytic activity are also less virulent. Once in the cytosol, the bacteria multiply with a doubling time of approximately 1 hour.

L. monocytogenes is able to move around in the cell by the recruitment of host cell actin; the key mediator of this intracellular movement is ActA, which is expressed on the bacterial surface in a polar fashion. In concert with several host cell proteins, an actin tail is assembled that propels the bacterium through the cytosol [20]. When the bacterium reaches the cell membrane, protrusions are formed, and the bacterium can be taken up by a neighbouring cell ("cell-to-cell spread"), resulting in the formation of a double membrane vacuole. This membrane can again be disrupted, a

process that is dependent on LLO and plcB but also requires phospholipase C A (plcA) [14]. The bacterium can then replicate in the cytosol of the new host cell and start a new infection cycle.

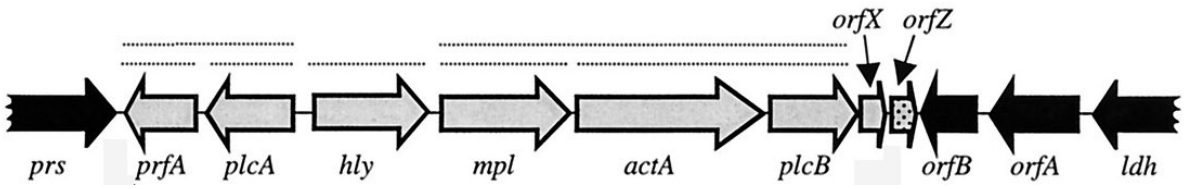

Fig. 1.4: Organization of the *L. monocytogenes* virulence gene cluster (LIPI-1). Genes belonging to LIPI-1 are in grey. Dotted lines above the LIPI-1 genes indicate known transcripts [175].

The virulence factors essential for this life cycle are regulated by prfA. The *prfA*, *hly*, *actA*, *plcA* and *plcB* genes are located in a virulence gene cluster (LIPI-1) on the bacterial chromosome (Fig. 1.4), while *inlA* and *inlB* are found in a distinct location, the so called internalin islet [175].

The course of a *Listeria monocytogenes* infection

Since contaminated food is the major source for *L. monocytogenes* infection, the gastrointestinal tract is thought to be the primary site of entry into the host [176]. Before reaching the intestine, the bacteria have to cross the adverse environment of the stomach. During this step, a significant number of bacteria may be already killed. Once in the intestine, the Listeria cross the intestinal barrier by a mechanism that is still controversially discussed. Data are available that suggest invasion of intestinal epithelial cells from the apical side [79; 132]. Other studies suggest that entry occurs via the highly phagocytic M cells overlaying the Peyer's Patches [74; 150]. Intestinal translocation of Listeria occurs without severe lesions in the gut of mice [103], suggesting that a phase of replication in the intestinal epithelium is not necessary for systemic infection. Indeed, the bacteria are translocated to deeper organs very rapidly. By using mutant strains lacking virulence factors like internalin A and B, LLO or ActA, or even the non-pathogenic species *L. inocua* it could be shown that translocation occurred at the same rate as with wild type *Listeria monocytogenes*

[130]. These findings favour the view that listerial translocation is a passive, non-specific process [176].

After crossing the intestinal barrier, the Listeria migrate rapidly via the lymph or blood to the mesenteric lymph nodes, the spleen and the liver [103; 130]. Experimental infections of mice via the intravenous route demonstrated that *L. monocytogenes* is rapidly cleared from the blood stream and taken up by resident macrophages in the spleen and liver [18; 23; 101]. Most of the bacterial load accumulates in the liver, presumably captured by Kupffer cells that line the hepatic sinusoids. Within the first 6 hrs of infection, most bacteria in the liver are killed by neutrophilic granulocytes [106]. The remaining bacteria start growing and proliferate in mouse organs for 2 – 5 days.

Listeria infection of hepatocytes, both *in vitro* and *in vivo*, results in apoptosis of these cells and the release of neutrophil chemotractants [135]. Neutrophils appear at the site of infection within the first 24 hrs, and their key role during Listeria infection was demonstrated by using monoclonal antibodies to deplete such cells [19; 24; 25; 133; 136]. Mice depleted in neutrophils succumbed to an early, lethal infection, with large numbers of bacteria found in the liver parenchyma. Neutrophils migrating to the liver remove apoptotic hepatocytes and destroy released bacteria.

The interaction between the Listeria, macrophages and neutrophils is critical for the host during the early phase of infection. Listeria infected macrophages produce TNF-α and IL-12, which synergize to cause IFN-γ secretion by NK cells. In turn, IFN-γ together with TNF-α leads to full activation of macrophages which then display increased levels of MHC class II molecules and become listericidal through the production of free radicals [4; 6]. The necessity of IFN-γ, TNF-α and IL-12 was shown by depletion experiments with monoclonal antibodies or the study of infections in appropriate knock out mice. Mice lacking any one of these cytokines quickly succumbed to a high dose infection (reviewed in [171; 172]).

The adaptive immune response to Listeria infection is dependent on T lymphocytes. Since the bacteria are found in the cytosol as well as the phagosomes of professional antigen-presenting cells, the listerial antigens are presented via the MHC class I and class II pathway. Therefore, both specific CD4 and CD8 T cells are induced during Listeria infection. Especially cytotoxic T cells are important for sterile clearance of the bacteria and provide a memory pool of T cells capable of a more rapid immune response upon a secondary infection [91; 101; 104].

Most studies concerned with the immunity to Listeria focused on the obvious role of T cells in the immune response against this intracellular pathogen. However, there are additional plasma-protein dependent mechanisms that are involved in protection against this intracellular pathogen: 1) opsonization of the bacterium in the extracellular space by complement activation, leading to increased phagocytosis; 2) antibody binding to key surface molecules, inhibiting entry into host cells; 3) antibody mediated neutralization of toxins produced by the pathogen in the extracellular environment; 4) activity of antibodies inside of pathogen containing phagocytes, e. g. in the phagosome (summarized in [37]).

Two studies have revealed a role for natural antibodies [114] and anti-LLO antibodies [36], respectively, in the context of Listeria infection. The first study showed that naïve C57/B6 mice have low but detectable levels of natural anti-Listeria IgM antibodies in their serum. Comparison of the early dissemination of bacteria in wild type C57/B6 mice and antibody lacking μMT mice after a high dose intravenous infection revealed 5-10 fold higher bacterial titres in liver, kidney and brain of mice lacking antibodies. In contrast, the bacterial titre in the spleen was 79-fold lower in these μMT mice as compared to wild type mice. The authors suggest that the microorganisms agglutinated by natural IgM were targeted to spleen macrophages due to their increased size. These results were obtained at 6 hrs post infection. Thus, it is not yet clear whether the observed differences have an influence on resistance at later time points of infection.

In the second study the authors used anti-LLO antibodies to treat mice before intraperitoneal infection with Listeria. This resulted in lower numbers of bacteria in the spleen and liver both at 6 hrs and two days post infection. Furthermore, increased survival after a lethal challenge was demonstrated. As described above, the reasons for this effect could be opsonization of the bacteria due to surface associated LLO or inactivation of the toxin outside the cell or even inside the phagocytic vacuole. The relevance of these findings for natural infection might be questioned, though, since extremely low LLO specific antibodies are found in mice, even after several rounds of infection [36], and the intracellular replication and cell-to-cell spread protect the bacteria from the humoral immune response of the host. In contrast, titres of antibodies against LLO and other Listeria specific antigens were high in Listeriosis patients and healthy persons that were exposed to Listeria, indicating a potential role for the humoral immune response against Listeria in humans [54].

Listeria monocytogenes mediated gene transfer

L. monocytogenes mediated gene transfer *in vitro* has been shown for a broad range of host cells, including cell lines of epithelial origin, murine primary macrophages and macrophage-like cell lines, as well as human dendritic cells [30; 31; 55; 60; 88; 122]. To initiate gene transfer from the bacteria to the host cell, lysis of the bacterium has to be induced. This is currently achieved by two different systems. The first system is based on an attenuated strain of *L. monocytogenes* which lacks the *actA*, *plcB* and *mpl* genes [59]. This strain escapes efficiently from the phagosome, but is unable to move inside the cell or to spread from cell to cell. Its attractive property is that it causes less inflammatory reactions *in vivo* than the wild type strain. This attenuated strain was equipped with a bacteriophage derived autolysin expressed under the control of the *actA* promoter that is only active in the cytosol of host cells. With these bacteria, different genes were transferred into murine macrophages and primary human dendritic cells [29; 47; 158]. *In vivo,* gene delivery was shown in a study where cotton rats were injected intraperitoneally with *L. monocytogenes* carrying a eukaryotic expression plasmid encoding GFP. Reporter gene expression was detected in a limited number of peritoneal macrophages 3 days after infection [158].
An alternative system has been established in our group, employing antibiotics to kill intracellular bacteria and to initiate gene transfer (Fig. 1.5) [60]. It employs wild type *L. monocytogenes* or *L. monocytogenes* hly W491A – a strain that produces a LLO variant that possesses only ~10% lytic activity compared to the wild type LLO – as carriers for eukaryotic expression plasmids. Attenuation of the LLO activity significantly enhanced transfection efficiencies [88].
After testing several different antibiotics and their effect on gene transfer, it was observed that a combination of penicillin and streptomycin was most efficient [88], indicating a mechanism for plasmid release that is dependent on the efficient lysis of the bacterial cell wall. Using this system, we could demonstrate transfer of different reporter genes to a variety of cell lines, and the functional transfer of the *cftr* cDNA and a cDNA encoding a fusion protein of CFTR and GFP to CHO-K1 and HEp-2 cells [88; 188].

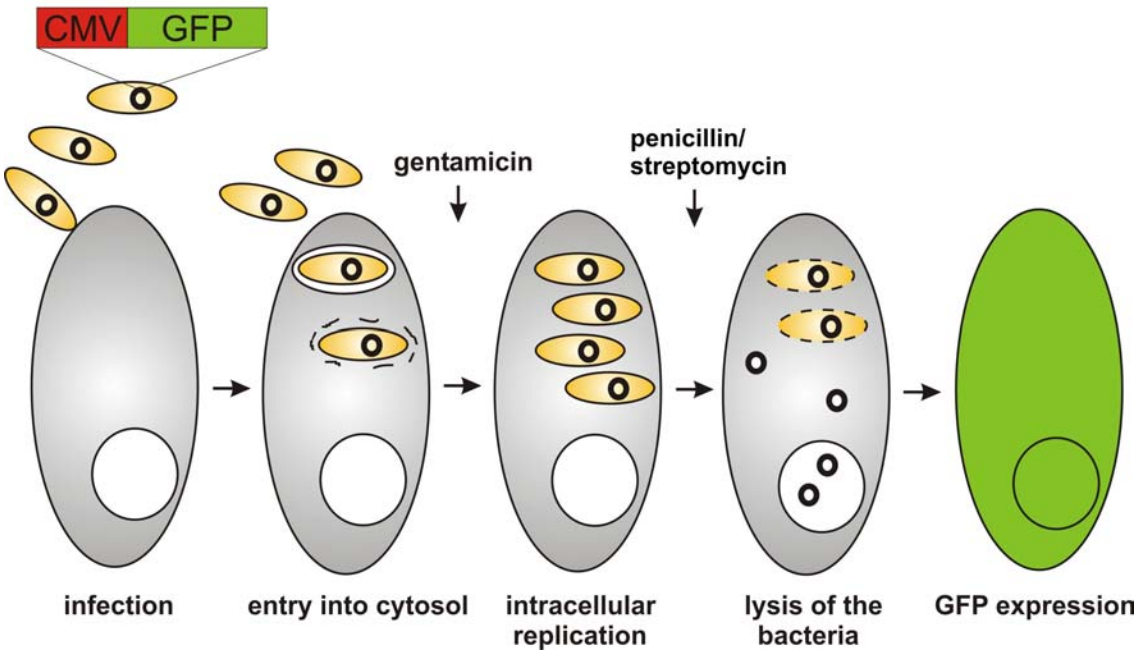

Fig. 1.5: Schematic illustration of the *in vitro* gene transfer system used in this work. After infection, extracellular bacteria are killed by addition of gentamicin. Subsequently, intracellular bacteria are allowed to replicate and spread to neighbouring cells. Then, intracellular bacteria are lysed by addition of penicillin/ streptomycin. Upon lysis, the expression plasmids are released and transgene expression (exemplarily shown here for GFP) can be analyzed.

In a recent study a vector/ carrier combination was presented in which the plasmid is stabilized in an antibiotic-free manner by a balanced suicide system [178]. The authors constructed a strain that lacks the essential gene encoding D-alanine racemase, and used a plasmid which complemented the D-alanine racemase mutation and carried a reporter gene. The possibility to stabilize the plasmid without an antibiotic resistance gene further adds to the safety of *L. monocytogenes* as a DNA carrier.

So far, no significant *in vivo* transfer of genes using any of these Listeria-based systems has been observed, although specific immune responses against *Mycobacterium tuberculosis* were reported after vaccination of C57/B6 mice with a recombinant *L. monocytogenes* strain carrying an expression plasmid for mycobacterial antigens [107]. Therefore, systems based on *L. monocytogenes* as a carrier for gene transfer require further improvement.

1.7.2 *Shigella flexneri*

As an alternative to Listeria, Shigella might be used for gene transfer, since these bacteria share properties with Listeria, like the ability to escape from the phagosome or to spread from cell to cell. The genus *Shigella* is a group of Gram-negative, facultative anaerobic bacteria that are genetically closely related to the genus *Escherichia.* The *Shigella* genus comprises the four species *S. flexneri*, *S. boydii*, *S. sonnei* and *S. dysenteriae*, which are further divided into serotypes according to biochemical differences and variations of their O-antigen. *Shigella flexneri* is divided into 13 different serotypes. It causes an infectious disease known as shigellosis by invading the colonic and rectal epithelium of primates and humans. The resulting acute mucosal inflammation causes severe tissue damage and leads to the clinical symptoms of watery diarrhoea, severe abdominal pain and cramping, and eventually bloody mucoid stool. Approximately 165 million people suffer from shigellosis each year, 1.1 million cases resulting in death. The vast majority of these cases occur in developing countries, and 69% of all patients are children under the age of five [73]. Transmission of the pathogen usually takes place via the faecal-oral route, which is augmented by poor hygienic conditions and close personal contact in developing countries. Mice are naturally not infected by this bacterium.

Intracellular life cycle

Shigella preferentially enters epithelial cells via their basolateral membrane [111]. However, the exact reason for that remains unclear. The mucin layer of colonic epithelial cells might act as a physical barrier and prevent close contact of the bacteria to the host cell, which is important for Shigella entry into epithelial cells. On the other hand, the basolateral membrane might display molecules that are not present on the apical side of the cell but are used as receptors for the Mxi/Spa secretion system. Close contact of this bacterial component to the host cell is essential for the induction of the first steps of the invasion process. Interactions between Shigella components and basolateral membrane proteins have been shown, including binding of Ipa proteins (see below) to $\alpha5\beta1$ integrin [179] and binding of IpaB to CD44 [155]. Both $\alpha5\beta1$ integrin and CD44 can act as cytoskeleton linkers, suggesting that they might take part in the reorganization of the actin skeleton (“membrane ruffling”) seen during uptake of Shigella. It also has been shown that

adhesion of Shigella to epithelial cells is dependent on the length and presence of the O-antigen [85]. These results suggest a potential involvement of LPS receptors like CD14 or Toll-like receptors (TLRs) in the entry process, both of which are found on the basolateral membrane of epithelial cells [2; 65].

The actual uptake of the bacterium by the cell occurs in a macropinocytotic process, where *S. flexneri* induced rearrangements of the host cell cytoskeleton engulf the bacterium and form a vacuole. Two loci on the 220 kb virulence plasmid of *S. flexneri* are crucial for this process, the *ipa* locus and the *mxi-spa* locus. The *ipa* locus encodes the proteins IpaA, IpaB, IpaC and IpaD, which are the effector molecules of bacterial entry into the host cell. These proteins are secreted by the type-III secretion system encoded by the *mxi-spa* locus.

The detailed mechanisms by which Ipa proteins trigger uptake of the bacterium are not completely understood. Secretion of the Ipa proteins is induced by contact with the host cell [105; 117]. Subsequently, the N-terminus of IpaC binds IpaB, leading to the insertion of the two hydrophobic proteins into the host cell membrane to form a pore [10; 57]. Other effector proteins are also thought to access the host cell cytoplasm via this pore. Several of the Ipa proteins can then interact with cytoskeleton associated proteins, leading to rearrangements of the actin filaments, formation of membrane extensions and eventually internalization of *S. flexneri* within a macropinocytic vacuole.

This vacuole is rapidly lysed by IpaB, which acts as membranolytic toxin, leading to the release of Shigella into the host cell cytosol [61]. IpaC may also be involved in this lytic process by inserting its hydrophobic regions into the membrane [28; 89]. Once in the cytosol, Shigella can replicate with an *in vitro* doubling time of 40 minutes.

mxiE, a gene located in the *mxi/spa* locus, is a transcriptional regulator of several putative virulence factors and is up-regulated only in the cytosol of the host cell. This suggests a role for *mxiE* as a general regulator for virulence genes involved in the post-invasion steps of infection [78].

S. flexneri, like *L. monocytogenes*, is able to recruit the host cell's actin to move around in the cell and also spread to neighbouring cells. The outer membrane protein IcsA is expressed on one pole of the bacterium and mediates actin recruitment [51]. DegP also seems to be required for efficient intracellular spread and unipolar expression of IcsA, although its exact function is still unknown [131]. IcsA interacts

specifically with neural Wiskott-Aldrich syndrome protein (N-WASP) and possibly with vinculin [50; 98; 163; 166], thus stimulating actin polymerization mediated by the actin-related protein (Arp) 2/3 complex [38; 164]. This actin polymerization propels the bacterium through the cytosol of the host cell.

When the moving bacterium reaches the cell membrane, a protrusion is formed which is actively taken up by the neighbouring cell [109]. The new double membrane vacuole is lysed by secreted IpaB and IpaC [116]. Another protein, VacJ, has been shown to be also essential for releasing Shigella into cytoplasm of the "secondary" cell [165]. Here, *S. flexneri* can replicate again and start a new infection cycle. In that way, it can move within the intestinal epithelial layer without being exposed directly to the humoral effector molecules of the immune system of the host.

The course of a *Shigella flexneri* infection

S. flexneri is highly infectious, and as little as 100 bacteria can cause disease in adult humans [33]. During uptake via the oral route, Shigella can survive the acidity of the stomach by up-regulating acid resistance genes. Once in the intestine, *Shigella flexneri* is taken up by highly phagocytic M cells that are concentrated in the epithelium above the mucosa-associated lymphoid tissues (reviewed in Fig. 1.6), but are also found all over the small intestine. These cells sample antigen from the lumen of the gut and pass it to the basolateral side of the epithelium by transcytosis, where lymphocytes and macrophages reside to take up antigen and raise a mucosal immune response. By this mechanism, *S. flexneri* crosses the epithelial barrier and gains access to macrophages and the basolateral membrane of epithelial cells. Furthermore, *S. flexneri* has recently been shown to be able to cross a model intestinal barrier by interfering with tight junction associated proteins of human intestinal epithelial cells, thereby using an M cell independent way of entry (Fig. 1.6; [138]).

Once entered, the bacteria replicate inside the cells and spread to neighbouring cells (as described above). As Shigella infection proceeds, infected macrophages undergo apoptosis, which leads to production of high amounts of interleukin-1 (IL-1). IL-1 is important in induction of inflammation and recruits polymorphonuclear cells (PMN) cells to the site of infection. Furthermore, IL-8 is induced by infection of epithelial cells, again attracting PMN cells. Consequently, the integrity of the epithelial lining is

disrupted to allow the PMN cells to transmigrate to the luminal side, at the same time facilitating entry of luminal bacteria through the epithelial barrier (Fig. 1.6). The recruitment of PMN cells is crucial for the generation of shigellosis-associated inflammation and tissue damage, as was shown in studies by inhibiting either IL-1 or IL-8 in rabbits [140; 141].

When CD18, an adhesion molecule used by PMN cells during migration, was blocked, tissue damage and bacterial invasion was diminished [127]. It is somehow stunning that the same PMN cells promote the local spread of the bacteria by disrupting the epithelial layer and at the same time also restrict the infection to the submucosa and prevent systemic dissemination.

The inflammation can persist in the gut for over a month. Resident macrophages and infiltrating monocytes are unable to efficiently kill the bacteria in their phagosomes but instead undergo apoptosis that leads to the expression of IL-18 [58; 191]. This cytokine induces the secretion of IFN-γ by NK cells and T cells [9] which in turn activates more macrophages and fibroblasts, possibly inhibits bacterial replication within epithelial cells and finally promotes bacterial clearance [180]. Only invading PMN cells are capable of killing Shigella in their phagosomes and play a crucial role in controlling the infection.

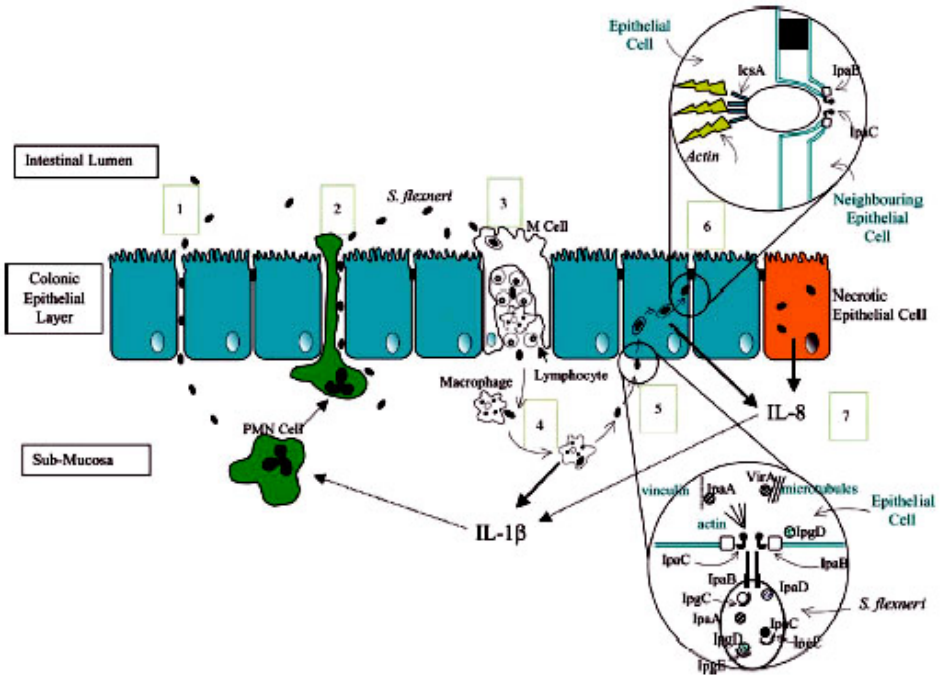

Fig. 1.6: Illustration of *S. flexneri* infection of the intestine and intracellular life cycle (from [73]). See text for more details.

Very little is known about the cellular immune response to *S. flexneri*. T cell activation was observed in shigellosis patients, and cytokines induced by vaccination against *S. flexneri* suggest Th1 and Th2 responses [68-70; 86; 174; 190]. Other studies conducted in a mouse model defective of T cells did not support the view that T cell responses are essential for protection against *S. flexneri* [181].

More data are available for the humoral immune response against Shigella infection from serological studies in infected humans and from animal experiments. These data suggest a crucial role for humoral responses in protective immunity. As serum IgG, IgM and secretory IgA have all been detected in infected individuals it seems that both systemic and mucosal immunity is activated. The main specificities of the antibodies are directed against serotype specific LPS and some virulence plasmid encoded proteins, including the Ipa proteins. The overall importance of an antibody response has recently been shown by a study which showed that a reduced and delayed antibody response in children compared to adult patients is the likely cause for increased susceptibility of infants to Shigella infection [134].

Shigella flexneri mediated gene transfer

S. flexneri is known to infect intestinal epithelial cells and macrophages in humans after oral uptake. However, as mentioned above, this pathogen is not invasive in the murine intestine, therefore limiting the experimental evaluation of this carrier to nasal challenge models. *S. flexneri* has so far been used mainly as DNA carrier in vaccination protocols against viral antigens. The first studies were undertaken with an attenuated strain of *S. flexneri* bearing a mutation in the *asd* gene, which encodes an essential compound for cell wall synthesis, as described before. These bacteria were shown to be able to deliver a eukaryotic expression plasmid encoding *E. coli* β-galactosidase to mammalian cells [153]. Also, humoral and cellular immune responses against β-galactosidase were raised after nasal inoculation of mice with these bacteria [154].

Intranasal immunization of mice with Shigella harbouring an expression plasmid encoding for HIV gp120 induced specific T cell responses and protected the mice against a challenge with a recombinant vaccinia virus [146]. When strains of Shigella, *S. typhimurium* and *S. typhi* carrying expression plasmids for viral antigens were directly compared by intranasal application, the Shigella strain proved to be superior

for the induction of antigen-specific CD8 T cell responses. Intramuscular injection of DNA showed similar cellular responses as the Shigella strain [177]. Regarding the humoral immune response, the DNA vaccine induced strong, specific IgG response in serum, compared to a superior mucosal antigen-specific IgA response induced by the Shigella vector. Other Shigella strains were used in combined prime-boost immunization protocols against HIV antigens together with naked DNA or viral vectors [146; 185]. Shigella were also used to deliver DNA vaccines encoding measles virus (MV) proteins to mice and cotton rats, leading to antigen-specific cellular responses and low levels of antibodies in serum [41; 123]. Thus, despite of the limits in model organisms *S. flexneri* appears to be an efficient vector for gene transfer *in vivo*.

In this work, *Shigella flexneri* is used not as carrier for DNA vaccines, but its ability to transfer DNA to tumour cells of epithelial origin is explored.

1.8 Aim of this work

Gene therapy is a powerful approach to cure or interfere with a wide range of diseases, including monogenic disorders, infectious diseases and cancer. However, in many cases the efficiency of gene transfer as well as the level and duration of expression were too low for clinical benefits, indicating the necessity for further improvement of the vectors and gene transfer systems used. Therefore, bacteria were introduced as alternatives to the vector systems used so far.

A *L. monocytogenes*-based *in vitro* gene transfer system that involves antibiotic-mediated killing of intracellular bacteria has been established and optimized by our group. In previous studies, this system allowed successful transfer of reporter genes as well as the functional transfer of a potentially therapeutic gene, *cftr*, to mammalian cells [88]. For the *in vivo* application of this transfer system, however, several obstacles have to be overcome, the most important one being efficiency of transfection.

In the first part of this work, therefore, the mechanism of “bacteriofection” was investigated in more detail. By identifying and eliminating the restrictions of this approach the transfer efficiencies should be significantly enhanced. Also, the fate of potentially co-transferred bacterial chromosomal DNA should be clarified, a safety issue that needed to be addressed.

In the second part, the use of bacteria as carriers for cancer gene therapy should be evaluated, using two different species, *Listeria monocytogenes* and *Shigella flexneri*. Our *in vitro* gene transfer system had to be adapted to the use of *S. flexneri* attenuated strains, and gene transfer to tumour cell lines *in vitro* by *S. flexneri* and *L. monocytogenes* needed to be demonstrated. Subsequently, a mouse tumour model should be employed to investigate the fate of the bacteria after infection via the intratumoural, intravenous and/ or intraperitoneal route. Finally, bacteria-mediated gene transfer to tumour tissue *in vivo* should be analyzed using different reporter genes.

2 Materials and Methods

2.1 Bacterial strains and growth conditions

The *Escherichia coli* (*E. coli*) strains TOP 10 and DH5α (Invitrogen, Groningen, The Netherlands) were used for general cloning. *E. coli* were grown in Luria Bertani (LB) broth or on LB-agar plates [139] at 37°C. Ampicillin (Sigma, Taufkirchen, Germany) was added at 100 µg/ ml kanamycin at 30 µg/ ml or erythromycin (Sigma) at 400 µg/ ml when required. *L. monocytogenes* strains EGDe [94] and hlyW491A [88] were grown in Brain Heart Infusion (BHI) broth or on BHI-agar plates (Difco, Detroit, MI) at 37°C. Medium was supplemented with 5 µg/ml erythromycin when indicated. Minimal medium for growth of *L. monocytogenes* W491A was freshly prepared and composed of the following [129]:

Compound	**Weight/ litre**
KH_2PO_4	6.56 g
Na_2HPO_4*$7H_2O$	30.96 g
$MgSO_4$*$7H_2O$	0.41 g
Ferric citrate	0.088 g
Glucose	10 g
L-Leucine	0.1 g
L-Isoleucine	0.1 g
L-Valine	0.1 g
L-Methionine	0.1 g
L-Arginine	0.1 g
L-Cysteine	0.1 g
L-Glutamine	0.6 g
Riboflavine	5 mg
Thiamine	1 mg
Biotin	0.5 mg
Thioctic acid	5 µg

S. flexneri strains ΔaroD and Δdap were grown in Tryptic Soy Broth (TSB) or on TSB-agar plates (Becton Dickinson, Heidelberg, Germany) supplemented with 200 µM congo red (Serva, Heidelberg, Germany) at 37°C. Medium for *S. flexneri* Δdap was

additionally supplemented with 100 µg/ ml DAP (Fluka, Buchs, Switzerland) and 30 µg/ ml kanamycin. Ampicillin was added at 50 µg/ ml when indicated.
Transformation of bacteria has been described elsewhere [120; 139].

2.2 Expression plasmids

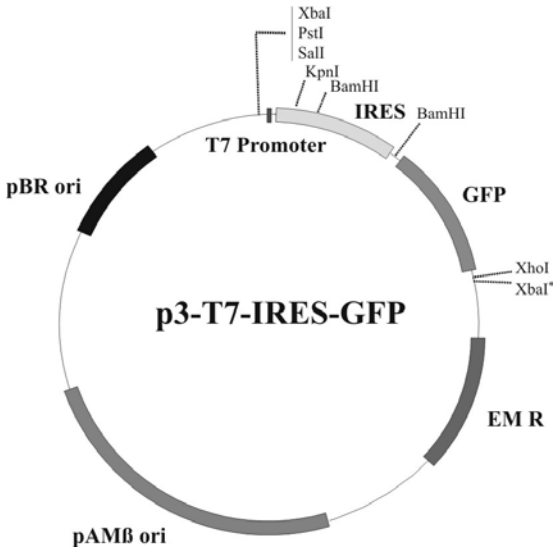

Fig. 2.1: Plasmid map of pERL3-T7-IRES-GFP. GFP expression is driven by the T7 promoter and an IRES element to provide cytosolic expression in host cells in the presence of T7 RNA polymerase. See text for construction details.

pERL3-T7-IRES-GFP

The IRES containing fragment from plasmid pMC-1 [32] was isolated by NotI digestion followed by Klenow treatment to generate blunt ends, and subsequent digestion with EcoRI. The vector pBluescript II KS(+) (Stratagene, La Jolla, CA) was treated in the same way, and the two fragments were ligated, resulting in the plasmid pBS-IRES. As the T7 promoter an adaptor fragment was used consisting of the oligonucleotides AAT TTC CCT ATA GTG AGT CGT ATT AG (EcoRI-pT7-SalI-V) and TCG ACT AAT ACG ACT CAC TAT AGG GA (EcoRI-pT7-SalI-R) that contained ends compatible with SalI and EcoRI. The sequence was designed such that the EcoRI site was destroyed upon ligation. This fragment was ligated into the corresponding restriction sites of pBS-IRES, giving rise to the plasmid pBS-T7-IRES.

The coding sequence of EGFP was obtained from pEGFP-1 (Clontech) by digestion with the enzymes SalI and Bsp120I and was inserted into the SalI and NotI sites of pBS-T7-IRES resulting in the plasmid pT7-IRES-GFP. The NotI restriction site was then destroyed by treatment with Klenow and a XhoI site was introduced using the linker CCCTCGAGGG. Then the T7 promoter controlled expression cassette was isolated by digestion with XhoI and PstI and ligated into the XhoI and PstI sites of the plasmid pERL-3 [60]. By this step, the kanamycin resistance gene was destroyed. The final plasmid was named pERL3-T7-IRES-GFP.

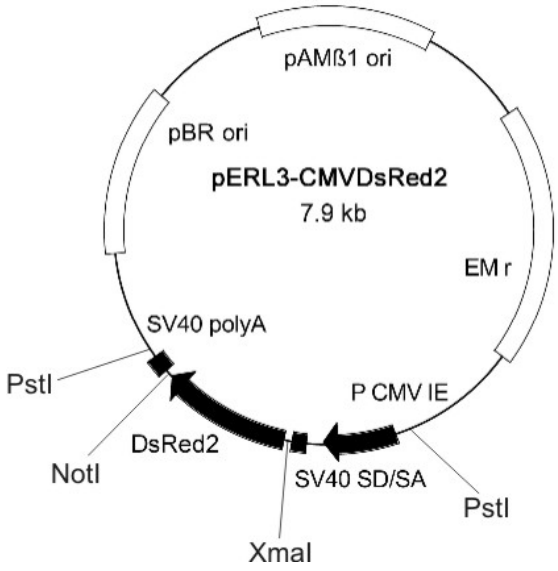

Fig. 2.2: Plasmid map of pERL3-CMV-DsRed2. This plasmid can be replicated in *E. coli* as well as in *L. monocytogenes* via the pBR or pAMβ1 ori, respectively. Expression of the red fluorescent protein DsRed2 is driven by the eukaryotic CMV promoter. See text for construction details.

pERL3-CMVDsRed2

The coding sequence DsRed2 was obtained by restriction digestion of the plasmid pDsRed2-N1 (Clontech) with XmaI and NotI. The coding sequence of EGFP was removed from the plasmid pERL3-CMVGFP [60] by digestion with the same enzymes and the DsRed2 containing fragment was ligated into these sites.

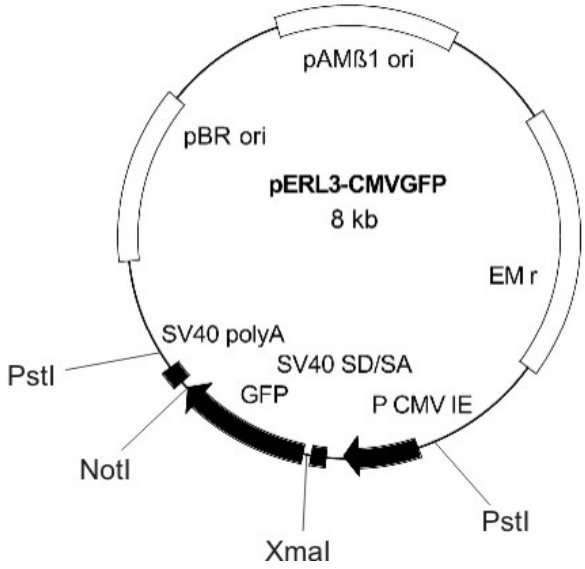

Fig. 2.3: Plasmid map of pERL3-CMV-GFP. This plasmid is equivalent to pERL3-CMV-DsRed2, but carries GFP as reporter gene. The construction of this plasmid has been described elsewhere [60].

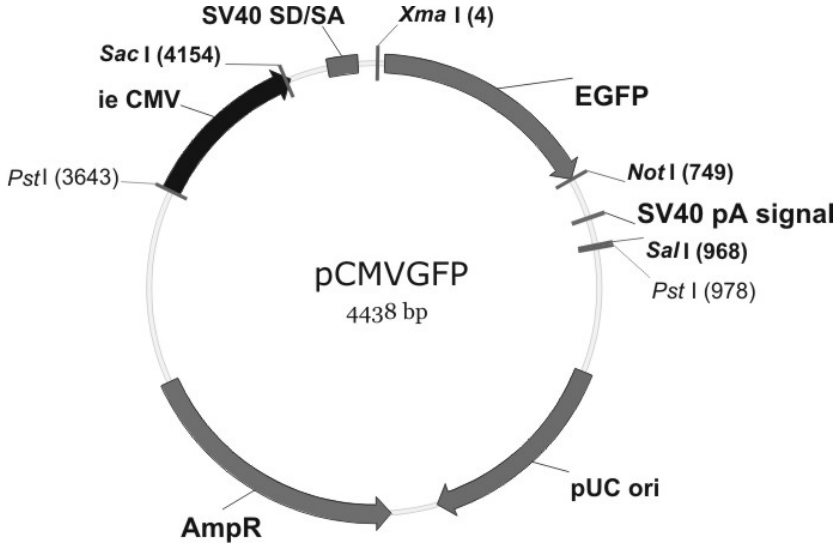

Fig. 2.4: Plasmid map of pCMV-GFP. β-galactosidase was removed from pCMV-β (Clontech) by digestion with NotI. GFP was obtained from pEGFP-1 (Clontech) by digestion with NotI and Bsp120I and the two fragments were ligated.

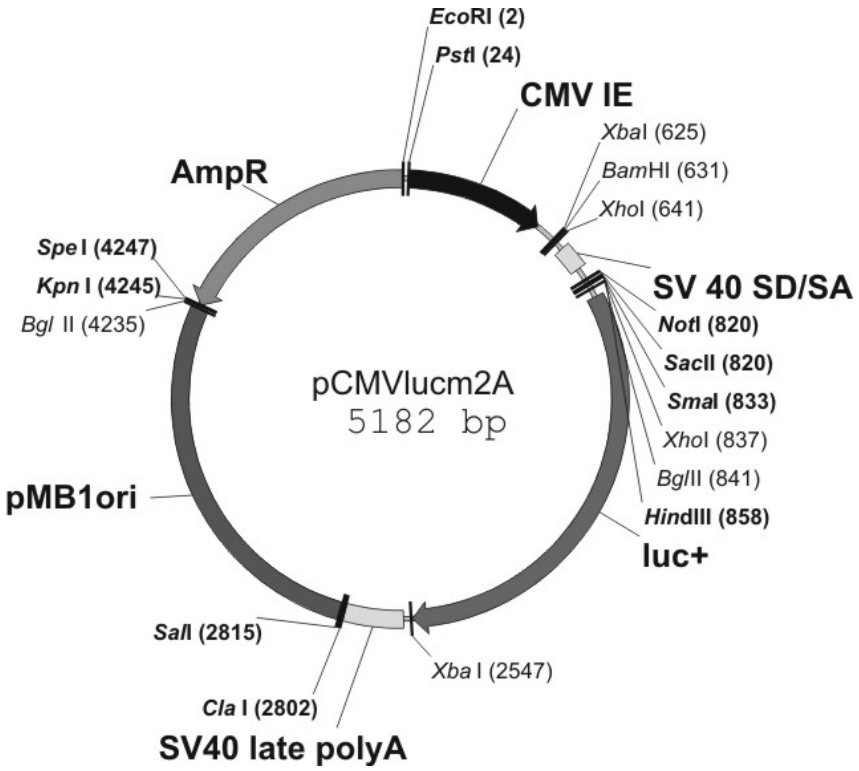

Fig. 2.5: Plasmid map of pCMV-luc-m2A. This plasmid confers expression of the firefly luciferase gene via the eukaryotic CMV promoter. The luciferase gene was isolated from pGL-3 Basic (Promega) and cloned into pCMVβ-m2A [5] via SmaI and SalI.

2.3 Cell lines

BHK-21 (ATCC CCL-10), TS/A [112], CT26 (ATCC CRL-2638), CHO-K1 cells (ATCC CCL-61) and HEp-2 cells (ATCC CCL-23) were cultured in IMDM (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Integro, Zaandam, The Netherlands) and 0.25 mM β-mercaptoethanol (Serva, Heidelberg, Germany). The cell line ST31-7 [88] was grown in the same medium but containing additional G418 (1 mg/ml; Gibco BRL). Cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere.

The clones CHO-GFPCFTR$^+$.1 and .2 stably expressing GFP-CFTR were generated by transfecting the plasmid pERL3-CMVGFPCFTR-neo into CHO-K1 cells using the method of calcium phosphate precipitation [139]. CHO-K1 transfectants were selected by growth in medium supplemented with G418 (1 mg/ml; Gibco BRL) and were cloned by sorting of single, GFP-positive cells using a FACSVantage (Becton Dickinson, Heidelberg, Germany) and screened by fluorescence microscopy.

2.4 *L. monocytogenes*- and *S. flexneri*-mediated transfer of eukaryotic expression plasmids to mammalian cells

Cells were seeded 24 hrs before infection in 24-well plates at a density of $5x10^4$ cells/ well, resulting in approximately $1x10^5$ cells/ well at the time of infection. Overnight cultures of *L. monocytogenes* and *S. flexneri* strains were diluted 1:50 in the corresponding medium and grown for 3-4 hrs and 2.5 hrs, respectively. When grown in minimal medium, *L. monocytogenes* from an overnight culture were washed twice with minimal medium, diluted 1:50 in minimal medium and incubated for 14 hrs. After washing once with PBS, bacteria were added to cell cultures at the indicated multiplicity of infection (MOI) and in a total volume of 200 µl medium. For *S. flexneri* ∆dap, PBS and culture medium was supplemented with 100 µg/ ml DAP. Subsequently, infection cultures were centrifuged to enhance invasion and incubated for 1.5 -2 hrs at 37°C. Cells were then washed twice with PBS and cultured in medium supplemented with 50 µg/ml gentamicin to kill the remaining extracellular bacteria. In case of Listeria, after additional 4 hrs of incubation at 37°C, intracellular bacteria were lysed by exchanging the gentamicin-containing medium for fresh medium supplemented with penicillin G (100 U/ ml) and streptomycin (100 µg/ ml) (Cytogen, Ober-Mörlen, Germany). Cells were incubated for the indicated time periods at 37°C and finally transient transgene expression was analyzed or cells were processed for electron microscopy or DNA isolation.

To detect GFP or DsRed expression by fluorescence microscopy, cells grown on coverslips were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Coverslips were mounted in Fluoprep (bioMérieux, Marcy l'Etoile, France) and preparations examined by epifluorescence microscopy using an Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat 100x/1.40 NA oil immersion objective. Images were recorded with a cooled, back-illuminated CCD camera (TE/CCD-1000 TKB; Princeton Instruments, Trenton, NJ). Flow cytometry for quantitation of GFP- or DsRed-expressing cells was performed with trypsinized, unfixed cells using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and CellQuest software (Becton Dickinson). The Dual Glo luciferase assay system (Promega, Mannheim, Germany) was used for analysis of luciferase expression according to the manufacturer's recommendations. 10 µl of the

each sample were measured and luminescence was recorded for 10 seconds using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.5 Transmission electron microscopy

For morphological analysis, the infected HEp-2 monolayers were fixed with 2% glutaraldehyde and 5% formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.9 M sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, pH 6.9) overnight on ice, washed three times with cacodylate buffer, treated with 1% aqueous osmium tetroxide for 1 h at room temperature and washed with cacodylate buffer. Samples were then dehydrated with a graded series of acetone (10, 30, 50%) on ice for 30 min for each step. Then, samples were left in 70% acetone with 0.5% uranyl acetate overnight, and further dehydrated with 90% and 100 % acetone. Infiltration of the samples was done with the low viscosity epoxy resin according to the described procedure by Spurr [159]. Finally, samples were placed into gelatine capsules, filled with pure resin and polymerized for 8 h at 70°C. Ultrathin sections were cut with a diamond knife and sections picked up with copper grids (300 mesh). Counter-staining of the sections was performed with lead-citrate for 2 min. After air-drying, samples were examined in a Zeiss EM 910 transmission electron microscope at an acceleration voltage of 80 kV.

2.6 Infection of tumour bearing BALB/c mice with *L. monocytogenes* and *S. flexneri*

To establish a subcutaneous tumour in BALB/c mice, CT26 or TS/A cells were harvested by trypsin treatment, washed once with PBS and resuspended in PBS to obtain a single cell solution of 10^6 – 10^7 cells/ ml. 100 µl were injected subcutaneously into each mouse. Infection was carried out at day 10 after tumour transplantation, when tumours were 5 – 8 mm in diameter.

Bacteria were grown overnight and diluted 1:50 in supplemented TSB medium for Shigella or minimal medium for Listeria (see 2.1). Bacterial cultures were grown to an optic density at 600 nm (OD_{600}) of ~0.8. Bacteria were then washed as described above and resuspended in PBS or PBS + DAP for *S. flexneri* ∆dap. Mice were infected with 10^6 to 10^8 CFU, as described in the figure legends. Intravenous and

intraperitoneal injections were performed in a total volume of 100 µl per mouse, while for intratumoural injection 10 µl per mouse were used. Transgene expression in the tumour as well as the number of viable bacteria in the different organs and in tumour tissues was determined at indicated time points by plating on appropriate plates.

To analyze GFP expression in tumour cells, solid tumours were excised from the mice. The tissue was first disrupted in cell culture medium using tweezers with sharp ends. Subsequently, cells were passed through a cell strainer (100 µm; BD Biosciences, Erembodegem, Belgium) to obtain single cell suspensions, which were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and CellQuest software (Becton Dickinson). To analyze luciferase activity, solid tumours were homogenized in 1 ml PBS supplemented with 0.2 % Igepal (Sigma). 125 µl of 5xPLB buffer from the Dual Glo luciferase assay system (Promega, Mannheim, Germany) were added to 500 µl of tumour homogenate and incubated for 15 min at room temperature. The sample was centrifuged for 1 min at full speed in a table top centrifuge and 10 µl of the supernatants were used to determine luciferase activity as described above.

To establish the number of GR-1 positive cells in the tumours of *L. monocytogenes* infected cells, cells were stained in 96 well plates (CellStar, Greiner Labortechnik, Frickenhausen, Germany). Single cell suspensions were prepared in FACS buffer (2% FCS, 2mM EDTA and 0,1% azid in PBS) at a density $5x10^5$ cell per well. Cells were first treated with mouse serum for 15 min on ice, to block Fc receptors, then stained for 15 min on ice, using a biotinylated rat-anti-mouse GR-1 antibody (Pharmingen, San Diego, USA), and washed with FACS buffer. The biotinylated antibody was revealed using streptavidin conjugated with phycoerythrin (Pharmingen, San Diego, CA), for 10 min on ice. After washing and staining for dead cells with 1 µg/ml of propidium iodide (Sigma-Aldrich, Deisenhofen, Germany) flow cytometry was performed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Data were analyzed with CellQuestPro software (Becton Dickinson, Heidelberg, Germany).

2.7 Determination of viable bacteria in cell cultures and mouse tissues

The number of viable intracellular bacteria was estimated by plating lysates of *in vitro* infected cells at different time points after infection. Cells were lysed by addition of 0.1% Triton-X 100 (Serva) in water and incubation for 15 minutes at room temperature. Serial dilutions of the lysates were prepared and aliquots were plated onto BHI or TSB agar plates, supplemented as described above. The number of colony forming units (CFU) was determined and the number of viable bacteria per well of a 24-well plate was calculated.
Alternatively, tissues of mice were homogenized in 1 ml (spleen and tumour) or 2 ml (liver) of PBS supplemented with 0.2 % Igepal (Sigma). Serial dilutions were plated on appropriate BHI or TSB plates as described above. The number of CFUs was determined and the number of viable bacteria per organ or tumour was calculated.

2.8 Isolation of DNA and Southern blotting

For the separation of nuclei and cytosol, infected cells were harvested by trypsin treatment 48 h after infection and single cell solutions in cold 5% citric acid were prepared at a density of 10^8 cells/ ml or less. To lyse the cells, cold 10% Igepal solution (Sigma) was then added to a final concentration of 0.5% while mixing the cells thoroughly. The cell suspension was underlayed with a cold solution of 30% sucrose/ 0.5% igepal/ 5% citric acid. The gradient was centrifuged for 10 min. at 2500 rpm in a Beckman GS-6KR centrifuge at 4°C. To obtain DNA present in the upper phase of the gradient, 0.3 M sodium acetate, 1 µg/ml glycogen (Böhringer, Mannheim, Germany) and 0.7 volumes isopropanol were added and incubated at –20°C overnight. The precipitate was spun down, washed once with 70% ethanol, air dried and resuspended in 10mM Tris-HCl (pH 7.6)/ 1mM EDTA (pH 8.0). The sediment containing the nuclei was resuspended in 100mM Tris-HCl (pH 8.5)/ 5mM EDTA (pH 8.0)/ 200mM NaCl/ 0.2% SDS. After addition of proteinase K (Sigma) at a final concentration of 0.4 mg/ml, the samples were incubated for 14-20 h at 54°C, DNA was precipitated by addition of 0.7 vol. of isopropanol and sedimentation at 13000 rpm in a table top centrifuge. The pellet was washed in 70% ethanol and after air drying resuspended in 10mM Tris-HCl (pH 7.6)/ 1mM EDTA (pH 8.0).

For southern blot analysis, indicated amounts of DNA from the nuclear fractions and the cytosolic fractions were digested with EcoRI. As a standard, different amounts of plasmid DNA (as indicated) were mixed with 5 µg of genomic DNA from uninfected HEp-2 cells and digested with EcoRI. Subsequently, the samples were separated by agarose gel electrophoresis. DNA was then transferred to a nylon membrane (Gene Screen plus, Perkin Elmer, Belgium) by capillary force under alkaline conditions. After the transfer, the membrane was washed for 1-2 minutes in 2xSSC [139]. DNA was fixed to the membrane by UV crosslinking with a Stratalinker 2400 (Stratagene, La Jolla, CA), applying 120 mJ.

The GFP specific probe was obtained by PCR using pERL3-CMVGFP as template and oligonucleotides GCGTGTCCGGCGAGGGCGAGGGCG as forward primer and GCCGAGAGTGATCCCGGCGGCGG as reverse primer. Radioactive labeling was carried out with the Ladderman Labeling Kit (Takara, Japan) according to the manufacturers recommendations. The membrane was then prehybridized with QuickHyb hybridization solution (Stratagene) for 45 minutes. Afterwards, the radioactive probe was added and the membrane was hybridized for 4 h at 65°C. After two short washings with 2xSSC/ 0.1% SDS and three times washing with 0.1xSSC/ 0.1% SDS for 10 minutes at 65°C, the membrane was exposed to a BioMax MS film (Kodak, Rochester, NY) for 1-2 h. Alternatively a phosphoimager Storm 860 (Molecular Dynamics/ Amersham, Freiburg, Germany) was used.

2.9 Isolation of genomic DNA and PCR analysis of bacterial DNA from bacteriofected CHO-K1 cells

Bacterial chromosomal DNA was isolated as previously described [44]. For isolation of DNA from CHO-K1 cells stably transfected by recombinant *L. monocytogenes,* or untreated controls, 10^7 cells were digested with Proteinase K (200µg/ml) for 48 h at 55°C in 1 ml Proteinase K buffer (10mM Tris HCl, 5mM EDTA, 1% SDS, 300mM NaAc, pH 8,0). The solution was transferred to 7 ml Vacutainer Brand SST tubes (Beckton Dickinson, Heidelberg, Germany), where the gel matrix completely seperates the organic and aqueous phase. 1 volume of a phenol/chloroform (1:1) mixture was added and the sample was mixed and centrifuged for 10 min at 1400 x g. To remove remaining traces of phenol, 1 volume of chloroform was added and the sample was again mixed and centrifuged. The solution was transferred to 2 ml

reaction tubes, DNA was precipitated with 1 volume of absolute ethanol, followed by centrifugation for 15 min at >20000 x g. After washing once with 70% ethanol, DNA was resuspended in H_2O.

For PCR experiments, the yield of DNA per cell was calculated and a DNA amount equivalent to 3000 cells was used as template. The following oligonucleotides (Invitrogen, Karlsruhe, Germany) flanking NotI restriction sites in the *L. monocytogenes* genome, were used as primers:

L.m.RS1-V: 5´-GGTAATCAAACGCCAGTAGGGACT-3´;
L.m.RS1-R: 5´-ATATGGCGTAGAAGGTTTC GCACC-3´;
L.m.RS2-V: 5´-AATCCGGTTCACCTGCACCAGTAA-3´;
L.m.RS2-R: 5´-AA TCAAGCGGCCGCTGATATTGCA-3´;
L.m.RS3-V: 5´-GGATTTTAAAGCAGTTGCAGC ACG-3´;
L.m.RS3-R: 5´-TGTCAAAGTCGCTAGAGTAGCGAA-3´;
L.m.RS4-V: 5´-ATGA ACACGATGGTATCGCCGTTC-3´;
L.m.RS4-R: 5´-AGGATGGATGCTGCTCGAACG TTT-3´;
L.m.RS5-V: 5´-GGTCACATTCTCTTCGGCTGCAAT-3´;
L.m.RS5-R: 5´-CTGGTTTTGAATGGCGTGAACCTG-3´;
L.m.RS6-V: 5´-CGTTTTATGAACGATTTGGA TGGC-3´;
L.m.RS6-R: 5´-CTCAATGCAATAGTACTCGACTCC-3´;
L.m.RS7-V: 5´-ATGCCTGATGCTAGGGTTGGTACA-3´;
L.m.RS7-R: 5´-TCCTATAAAACAAACGGCTCGCTG-3´.

As a control, GAP-DH specific primers with the following sequences were used:

GAPDH FOR: 5'-ATCTTCTTGTGCAGTGCCAGC-3'
GAPDH REV: 5'-ACTCCACGACATACTCAGCACC-3'

A standard PCR reaction was performed with 40 cycles of amplification using 60°C (*L. monocytogenes* specific primers) or 58°C (GAP-DH specific primers) as annealing temperature in a Hybaid Touchdown thermocycler. To determine the sensitivity of the PCR assay, the reaction was carried out with serial dilutions of purified genomic DNA of *L. monocytogenes* and DNA from 3000 CHO-K1 cells as a background. Under these conditions, specific bands could be detected with all primer pairs used, when an equivalent of 10 copies of the *L. monocytogenes* genome was present in the reaction (data not shown).

3 Results

3.1 Optimization and characterization of *L. monocytogenes*-mediated gene transfer *in vitro*

It is clear that Listeria-mediated gene transfer of therapeutic genes is in principle feasible [88]. In previous studies, first reporter genes and subsequently a cDNA encoding the cystic fibrosis transmembrane conductance regulator (CFTR) was functionally transferred to CHO-K1 and HEp-2 cells *in vitro* using *L. monocytogenes* [88]. As an extension, a simple reporter system for a facilitated detection of *in vivo* CFTR expression for future studies was established by fusing GFP to the N-terminus of CFTR (GFP-CFTR) [188]. The correct intracellular location and functionality of both CFTR and GFP-CFTR were shown by immunoblotting, fluorescence microscopy and whole cell patch clamp analysis. The number of CFTR channels per cell revealed by the latter technique was in a physiological range. However, the number of bacteriofected cells was too low to be of clinical benefit. To rationally improve the efficiency, it is necessary to understand the mechanism of bacteria-mediated DNA transfer in order to find the limiting step. Therefore, as a major part of this work the fate of the plasmid DNA after bacterial death was studied.

3.1.1 Invasion of host cells is not the major limitation

An obvious step limiting gene transfer efficiency could be the invasiveness of the bacteria. Thus, an increase in the number of bacteria taken up by the host cell and capable of transferring their plasmid load should enhance bacteriofection. Invasiveness of the strain *L. monocytogenes* hly W491A, which is secreting an attenuated listeriolysin [88], could be augmented by culturing the bacteria in minimal medium before infection. This leads to the up-regulation of virulence factors that are involved in the invasion process of the host cell [72]. Three different cell lines were bacteriofected and in parallel the number of intracellular bacteria was determined by plating at the start point of antibiotic treatment. Furthermore, the number of GFP expressing cells was established by flow cytometry at 48 hrs after initiation of bacteriofection.

As shown in Fig. 3.1, the number of intracellular bacteria was 5- to 6-fold higher at the beginning of antibiotic treatment when bacteria were grown in minimal medium compared to rich BHI medium (Fig. 3.1 A). However, the number of transfected cells had increased only 2- to 3-fold (Fig. 3.1 B). These results clearly show that invasion of the host cell is an important event during the process of bacteria-mediated gene transfer. However, it is not the only limiting factor, otherwise the increase in numbers of transfected cells should be directly correlated to the increase in numbers of intracellular bacteria.

3.1.2 A minor fraction of the intracellular carrier bacteria transfer plasmids to the nucleus of the host cell

These results stipulated to investigate further whether all bacteria that have entered the host cell are able to transfer their plasmid load to the nucleus. To this end, two different recombinant strains of *L. monocytogenes* were used harbouring the expression plasmids pERL3-CMVGFP, encoding the green fluorescent protein, and pERL3-CMVDsRed2 that encoded a red fluorescent protein. The hypothesis was that when all intracellularly lysed bacteria are able to transfer expression plasmids, a co-infection of HEp-2 cells with both carrier bacteria at high MOIs (740 for pERL3-CMVGFP and 640 for pERL3-CMV-DsRed2) should result in expression of both fluorescent proteins in all cells that could be transfected. This was clearly not the case, as shown by fluorescence microscopy 48 hrs after initiation of DNA transfer (Fig. 3.2 A). Similarly, when bacteriofected cells were analyzed by flow cytometry**,** only 6.3% of the cells were expressing both proteins (Fig. 3.2 B and C). Despite the extremely high MOIs used for both carrier bacteria, 18.1% of the bacteriofectants expressed GFP alone and 7.1% were single positive for DsRed2 (Fig. 3.2 B and C). Thus, only extremely few of the many carrier bacteria that infect a host cell are able to transfer their plasmid load to the nucleus of the host cell.

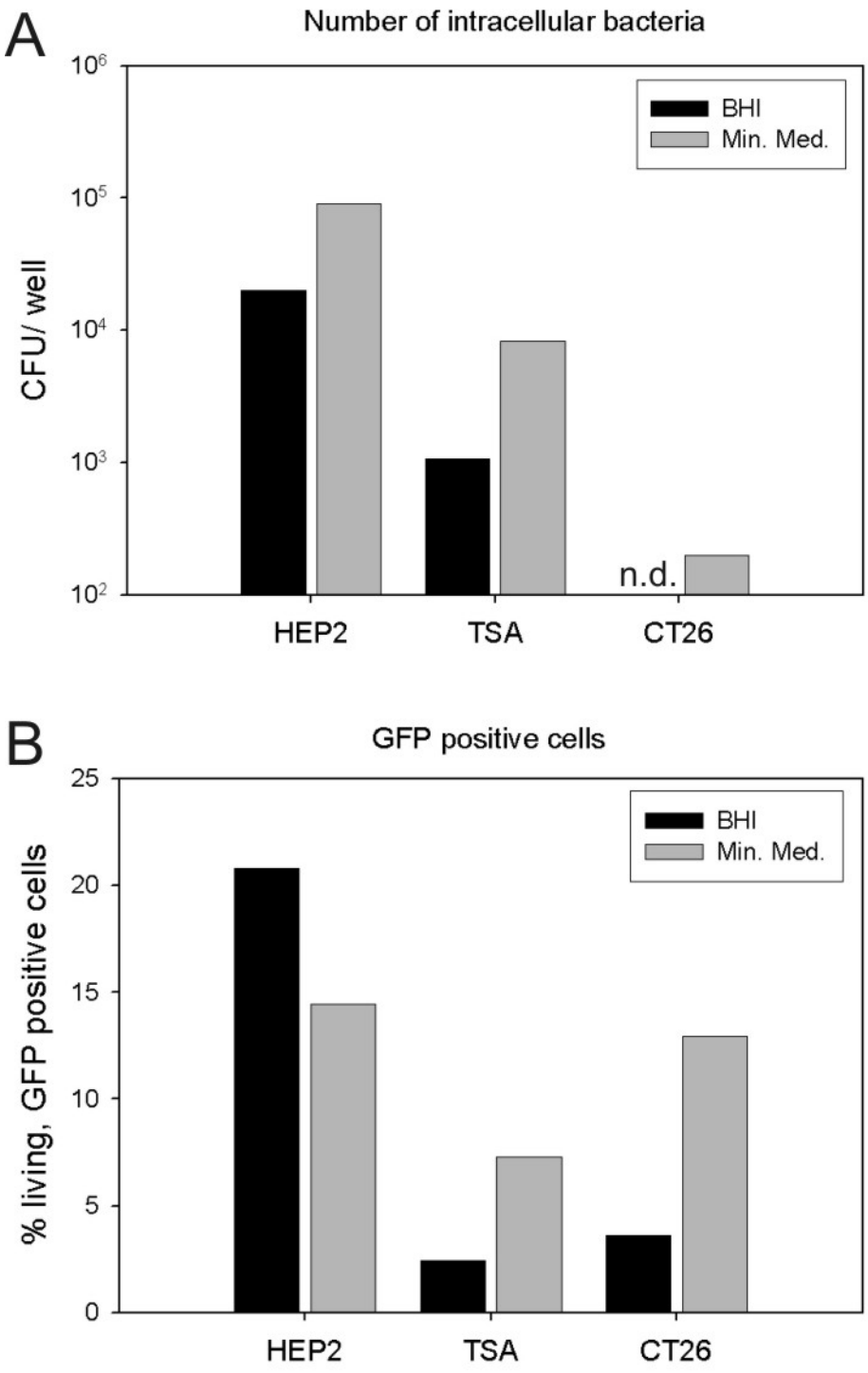

Fig. 3.1: Invasiveness of *L. monocytogenes* after culture in minimal medium and GFP expression after bacteriofection in HEp-2, TS/A and CT26 cells. Cells were infected with *L. monocytogenes* W491A carrying the expression plasmid pERL3-CMV-GFP at a MOI of 200 for HEp-2 and CT26 and 500 for TS/A, respectively. (**A**) The number of viable, intracellular bacteria was determined by plating cell lysates on BHI plates with 5 µg/ml erythromycin at 4 hrs p.i. (**B**) GFP expression was analyzed by flow cytometry 24 hrs after bacteriofection. Numbers represent the percentage of viable, GFP expressing cells. Data are representative for 1 out of 2 experiments. n.d.: not detectable

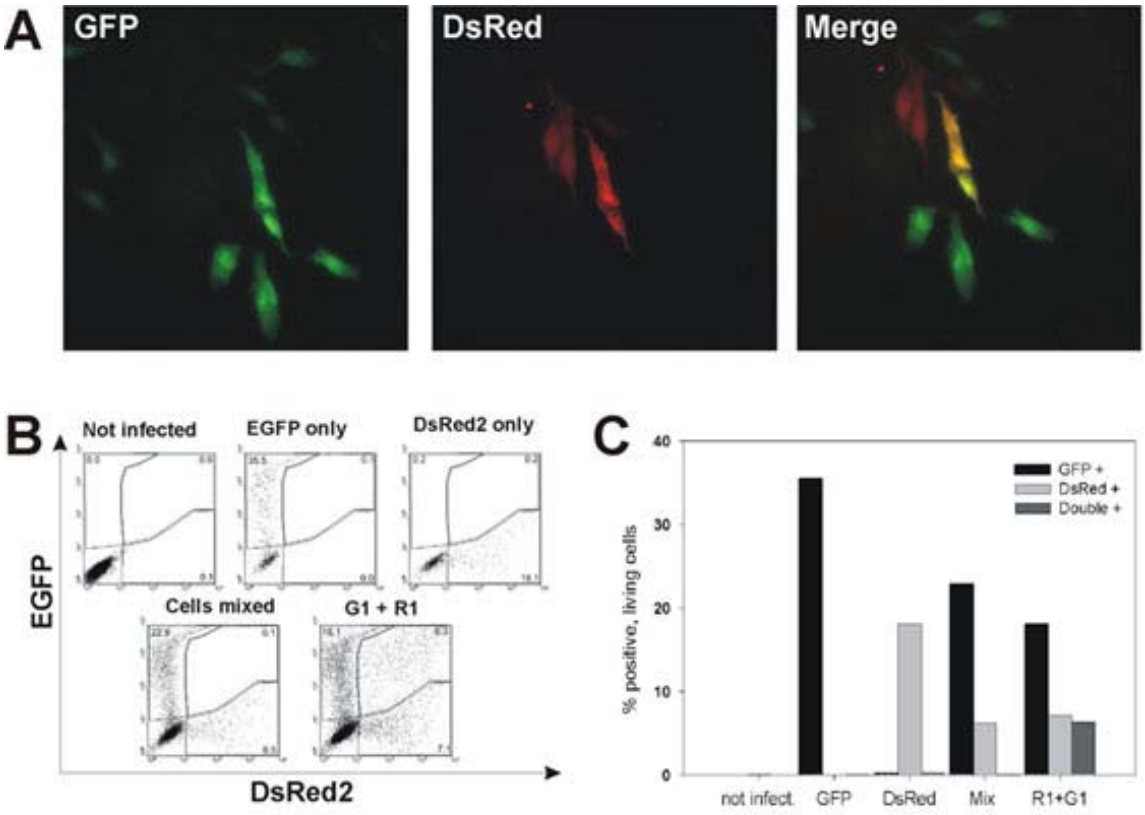

Fig. 3.2: Co-transfer of expression plasmids pERL3-CMVGFP and pERL3-CMVDsRed to HEp-2 cells by *L. monocytogenes*. Transgene expression was analyzed 48 hrs after bacteriofection. (**A**) Fluorescence micrographs of HEp-2 cells bacteriofected with MOIs of 12 for *L. monocytogenes* EGD carrying pERL3-CMVGFP and 19 for *L. monocytogenes* EGD carrying pERL3-CMVDsRed. (**B**) Flow cytometric analysis of co-transfected cells. HEp-2 cells were transfected by *L. monocytogenes* W491A carrying the plasmid pERL3-CMVGFP at a MOI of 740 (EGFP only), the plasmid pERL3-CMVDsRed2 at a MOI of 640 (DsRed2 only) or a mixture of both strains (G1 + R1). Uninfected cells were used as a negative control. For gating, cells from the single transfections were mixed (Cells mixed). The quantitation of this experiment is shown in (**C**). This experiment was repeated twice with similar results.

3.1.3 Plasmids liberated from carrier Listeria are not accessible in the cytosol of host cells

A possible explanation for this low transfection rate could be that the plasmids remain in the cytosol and are inefficiently transported to the nucleus. Therefore it was argued that strong expression should be possible when the complete expression process takes place exclusively in the cytosol. To address this point, the carrier bacteria were equipped with the plasmid pERL3-T7-IRES-GFP that consists of an EGFP encoding

cDNA under the control of the T7 promoter and an IRES from poliovirus type I. As host cell the BHK-21 derivative BSRT7/5 was used that constitutively expresses the T7 RNA polymerase. Under these circumstances, RNA should be transcribed from the plasmid by the T7 polymerase directly in the host cell cytosol and the IRES should render the translation of transgene Cap independent. A quick onset of transgene expression was expected and, if expression plasmids remained freely in the cytosol, a strongly elevated number of cells expressing the transgene.

To verify this idea, BSRT7/5 cells and BHK-21 cells were first transfected with pERL3-T7-IRES-GFP or pERL3-CMVGFP by the calcium phosphate precipitation method [139]. Flow cytometry revealed the earliest GFP positive BSRT7/5 cells transfected with pERL3-T7-IRES-GFP already after four hours. At this time point, no GFP expression was observed in BSRT7/5 cells transfected with pERL3-CMVGFP or BHK-21 cells transfected with pERL3-T7-IRES-GFP. Six hours after exposing the cells to the DNA precipitate a few GFP expressing cells were found in cells transfected with pERL3-CMVGFP (Fig. 3.3 A and C). At 24 hrs, BHK-21 cells transfected with pERL3-CMVGFP and BSRT7/5 cells transfected with pERL3-T7-IRES-GFP showed comparable numbers of GFP expressing cells. Expression from the T7 driven plasmids decreased already 48 hrs after transfection while the number of GFP positive BSRT7/5 cells transfected with pERL3-CMVGFP was low at 24 hrs but increased at 48 hrs after transfection.

When similar transfections were performed as bacteriofections using *L. monocytogenes* W491A carrying the corresponding plasmids, early onset of transgene expression in BSRT7/5 cells bacteriofected with pERL3-T7-IRES-GFP was no longer observed (Fig. 3.3 B and C). GFP positive cells were only found after 24 and 48 hrs in BHK-21 cells bacteriofected with pERL3-CMVGFP. Remarkably, transfection of BSRT7/5 cells with Listeria using pERL3-CMVGFP as expression plasmid led to a low transgene expression after 48 hrs. The cause of this effect could not be explained at the moment.

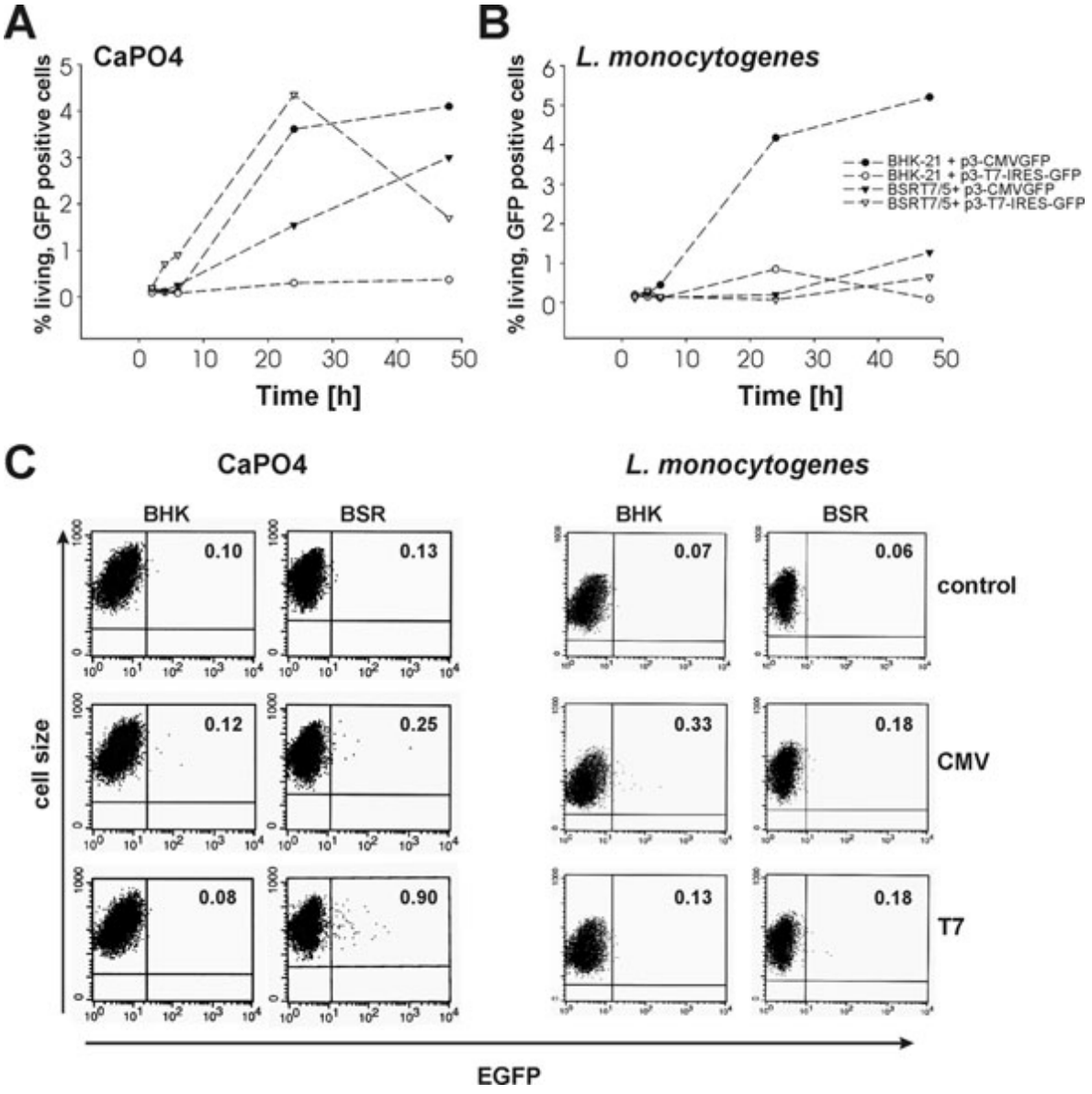

Fig. 3.3: Bacteriofection of BHK-21 and BSRT7/5 cells with a plasmid potentially expressed in the cytosol of host cells. BHK-21 and BSRT7/5 cells were transfected with the plasmids pERL3-CMVGFP and pERL3-T7-IRES-GFP, respectively, by either calcium phosphate precipitation (**A**) or by *L. monocytogenes* W491A mediated gene transfer (**B**). The number of living, GFP expressing cells was measured by flow cytometry at 2, 4, 6, 24 and 48 hrs after initiation of DNA transfer. (**C**) Flow cytometric analysis at 6 hrs post transfection. Dots in the upper right quadrant represent viable, GFP positive cells. BHK: BHK-21 cells, BSR: BSRT7/5 cells, control CaPO4: mock transfected cells, control *L. monocytogenes*: uninfected cells, CMV: transfection with plasmid pERL3-CMVGFP, T7: transfection with plasmid pERL3-T7-IRES-GFP. Significant expression could be detected only when the plasmid was transfected by calcium phosphate precipitation.

3.1.4 Bacteria are efficiently lysed and their content is released after treatment with antibiotics

The above results suggest that most plasmids introduced into the host cells by *L. monocytogenes* do not become accessible to the host cells expression mechanisms. They might be either associated with proteins, possibly of bacterial origin or they might be rapidly degraded. To distinguish between these possibilities the fate of the bacteria after treatment with antibiotics was first analyzed since the plasmids might remain associated with lysed bacteria. To this end, the number of viable bacteria during the course of the experiment was established by plating, as shown in Figure 3.4. Already 24 hrs after the addition of the antibiotics the number of colony-forming bacteria dramatically decreased and by 48 or 72 hrs hardly any platable bacteria were found.

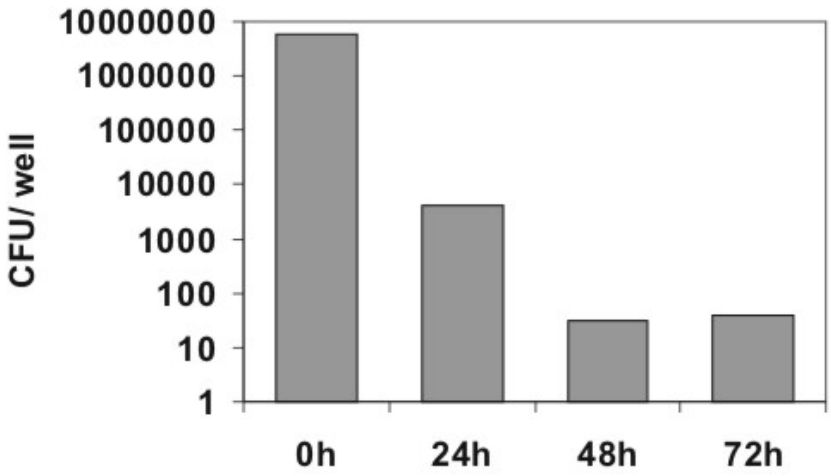

Fig. 3.4: Intracellular bacteria are efficiently killed by addition of antibiotics. HEp-2 cells were infected with *L. monocytogenes* W491A carrying the expression plasmid pERL3-CMVGFP at a MOI of 400. The number of viable intracellular bacteria was determined by plating cell lysates at different time points after addition of penicillin/ streptomycin. CFU, colony forming units.

This was confirmed using fluorescent live/dead staining for bacteria. Only a few live bacteria were detected at time points later than 24 hrs following initiation of antibiotic treatment (data not shown). Interestingly, also the number of detectable dead bacteria decreased dramatically under these circumstances (data not shown). Since the determination of dead bacteria is based on the staining of bacterial DNA with

propidium iodide that can be discerned as red fluorescent entities within the host cells cytosol, these data suggest that the content of the bacteria is no longer concentrated within the bacteria.

Therefore transmission electron microscopy was employed to directly examine the fate of the bacteria. Figure 3.5 A shows that, at the time of adding antibiotics, bacteria had an intact structure and were found either in typical phagocytic vacuoles or already in the cytosol. At 24 hrs, bacteria that are only partially filled with electron dense material could be found and some structures which obviously represent lysed bacteria or bacteria that are at the verge of releasing their content into the cytosol were observed (Fig 3.5 A arrows). Similar structures were detected at later time points, although they become rather rare at 48 and 72 hrs.

Interestingly, at 24 hrs and later, several bacteria are still found in vesicles. At this time all bacteria would have been expected to have escaped into the cytosol. Apparently, such vesicles fuse with lysosomes (Fig. 3.5 B arrows).

The electronmicrographs indicated that the bacterial content is released quickly into the cytosol of the host cell after adding the antibiotics. This suggests that bacterial proteins and other macromolecules should be found in the cytosol of the host cell, shortly after bacterial lysis. Therefore Listeria that heterologously expressed GFP under the control of a bacterial promoter (pERL3-cspl::gfp; [88]) were employed.

As shown in Fig. 3.6 B, already at the initiation of the antibiotics treatment 80% of infected cells contain significant amounts of GFP. However, GFP was exclusively associated with bacteria as observed by fluorescence microscopy (3.6 A). Roughly the same number of cells still contained GFP 48 hrs later (Fig. 3.6 B), but at this time point hardly any bacteria could be observed within the host cells. In addition, GFP was now evenly distributed over the entire host cell (data not shown). The fluorescence intensity observed after 48 hrs was similar to that detected at the time when antibiotic treatment was started, but was significant lower than seen after Listeria-mediated DNA transfer. Apparently, GFP expressed by the bacteria is released upon lysis of the bacteria into the cytosol of the host cell.

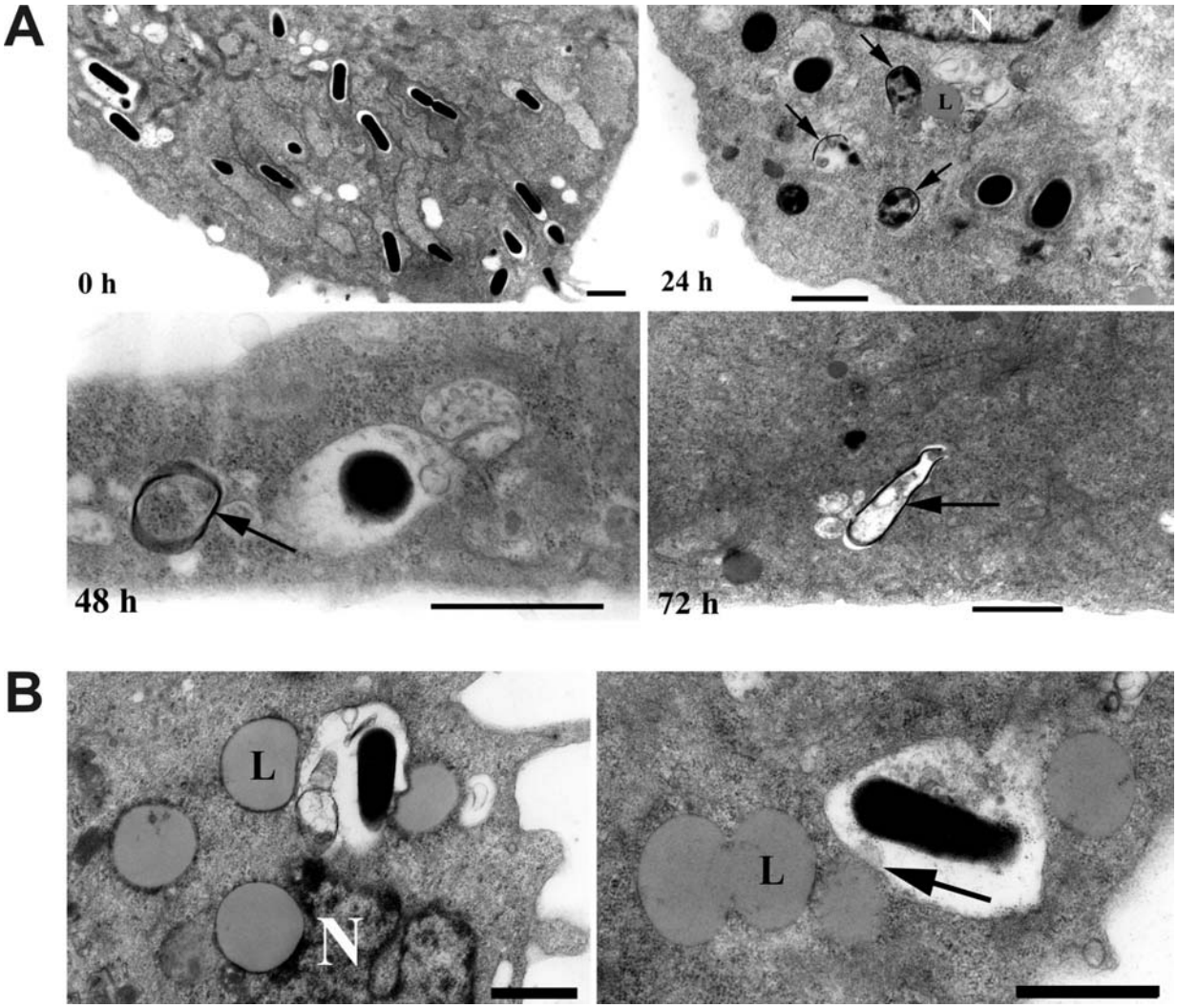

Fig. 3.5: Bacteria are efficiently lysed and their cell walls disrupted after antibiotic treatment. (**A**) Transmission electron micrographs of *L. monocytogenes* W491A infected HEp-2 cells at different time points after addition of antibiotics. Arrows indicate disrupted bacterial cell walls. Interestingly, despite of the long time after invasion many bacteria are found in vacuoles. In (**B**) the aggregation of lysosomes at sites of bacterial degradation is shown. The arrow indicates the fusion of a lysosome and a bacteria containing vesicle. Black bars correspond to 1 µm. L: Lysosome; N: Nucleus.

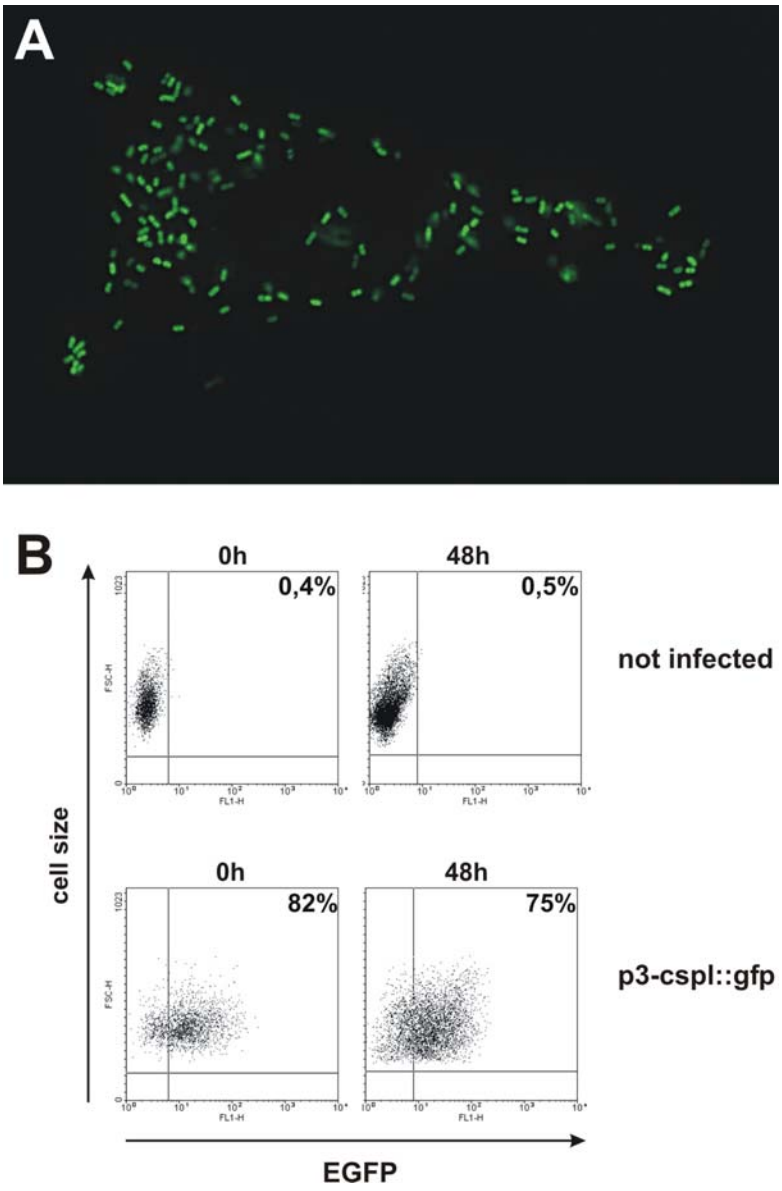

Fig. 3.6: Bacterial content is efficiently released into the host cell cytosol after bacterial lysis. (**A**) Fluorescent micrograph of a HEp-2 cell infected with *L. monocytogenes* W491A constitutively expressing GFP. Cells were analyzed just before start of antibiotic treatment. (**B**) Flow cytometric analysis of HEp-2 cells after infection with *L. monocytogenes* W491A constitutively expressing GFP (pERL3-cspl::gfp). 0h: cells were analyzed just before addition of antibiotics. 48h: cells were analyzed 48 hrs after the addition of antibiotics. Fluorescent micrographs of cells at that time point are not shown since the signal was too low to be recorded. Flow cytometry can reveal the presence of GFP since it displays an integration of the signal over the entire volume of the cell. Uninfected HEp-2 cells were used as negative control (not infected).

3.1.5 Transferred plasmids are associated with fast sedimenting entities

Following efficient release of the bacterial content the plasmid DNA should remain detectable in the cytosol of the host cell unless it is rapidly degraded. Therefore, HEp-2 cells were infected with *L. monocytogenes* W491A carrying the plasmids pERL3-CMVGFP and pERL3-CMVGFPCFTR-neo, respectively. The infected cells were lysed 48 hrs after infection and the lysate was centrifuged over a 30% sucrose cushion to separate the cell nuclei from soluble cytosolic compounds. DNA was isolated from both fractions and analyzed by Southern blotting. To estimate the number of plasmids in the nuclear and cytosolic fractions, three different amounts of pERL3-CMVGFP plasmid DNA mixed with HEp-2 genomic DNA were loaded onto the gel for comparison. Results are shown in Fig. 3.7. Signals specific for EGFP were found exclusively in the fraction sedimenting through the sucrose cushion when cells bacteriofected with pERL3-CMVGFP and pERL3-CMVGFPCFTR-neo were tested, respectively. No signals were detectable in the cytosolic fractions of these cells. Soluble plasmid DNA added to the cell lysate just before centrifugation was to a large extent found in the cytosolic fraction (data not shown). By comparing the intensity of signals from nuclear fractions with the plasmid standard, it was estimated that the number of plasmids recovered was in the range of the number of plasmids carried by the intracellular bacteria that were added at the time of infection. Keeping in mind the low transfection efficiency, it is highly unlikely that all plasmids were transferred into the nucleus. Therefore, the interpretation of the above result is taken as evidence that the plasmid DNA is associated with macromolecules that are of sufficient mass to sediment through the sucrose cushion during the separation of cell nuclei from cytosolic compounds. Furthermore, it clearly shows that the plasmid DNA is not degraded rapidly, but remains within the host cell for a considerable amount of time.

3.1.6 Integration of bacterial DNA into the host cell genome after *L. monocytogenes* mediated gene transfer

The rapid release of the complete bacterial content upon lysis of *L. monocytogenes* within eukaryotic cells, also results in the release of large quantities of bacterial chromosomal DNA. This could in turn result in transfer and subsequent integration into the host cell genome. To test this possibility, genomic DNA was isolated from different CHO-K1 derived cell lines and clones that had been bacteriofected with *L.*

monocytogenes. A PCR was performed using 7 primer pairs to scan and detect fragments of the listerial chromosome. The primer pairs that were selected did not show any amplification product with DNA from the parental CHO-K1 cells (data not shown). A DNA-amount equivalent to 3000 CHO-K1 genomes was tested. The sensitivity of this assay allowed reproducibly the detection of DNA-amounts equivalent to 10 *L. monocytogenes* genomes.

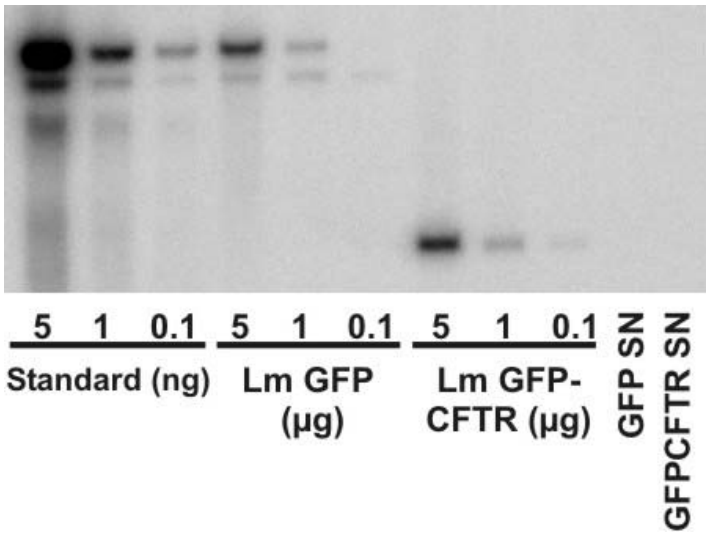

Fig. 3.7: Southern blot analysis of nuclear and cytosolic fractions of bacteriofected HEp-2 cells. Cells were lysed 48 hrs post infection with Igepal and fractionated by centrifugation over a 30% sucrose cushion. DNA was isolated by isopropanol precipitation Standard: pERL3-CMVGFP plasmid DNA mixed with HEp-2 genomic DNA as carrier DNA. Lm GFP: Nuclear fractions from HEp-2 cells transfected by *L. monocytogenes* W491A carrying the plasmid pERL3-CMVGFP. Lm GFP-CFTR: Nuclear fractions from HEp-2 cells transfected by *L. monocytogenes* W491A carrying the plasmid pERL3-CMVGFPCFTR-neo. GFP SN/ GFPCFTR SN: corresponding cytosolic fractions after concentration by isopropanol precipitation.

First, the cell line ST37, which was stably transfected with a GFP-CFTR encoding plasmid by *L. monocytogenes*, was tested. A specific signal was obtained for all 7 primer pairs (Fig. 3.8). When this cell line was further sorted for GFP expressing cells (ST37-S1), only 3 of the 7 bands remained detectable. Repeated sorting of medium and high fluorescent cells from the ST37-S1 population resulted in the two subpopuations ST37-S2H and ST37-S2M, but neither of them gave rise to specific amplification products. The same was true for the clone ST37-2H.43 derived from ST37-S2H and for 2 clones, ST37-2M.12 and ST37-2M.19, derived from the ST37-S2M line.

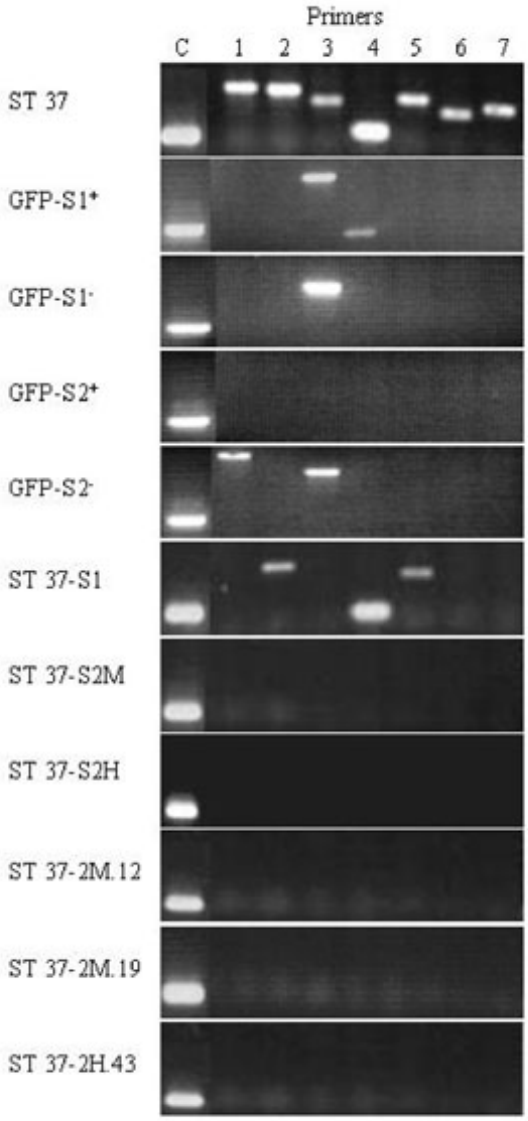

Fig. 3.8: Presence of DNA fragments derived from the bacterial chromosome in the genome of CHO-K1 cells after *L. monocytogenes*-mediated gene transfer. Primer pairs (1-7) were specific for *L. monocytogenes* genomic DNA or GAP-DH (C). All cell lines and clones were derived from CHO-K1 and stably transfected by *L. monocytogenes*-mediated gene transfer. ST37, bulk culture expressing GFP-CFTR; ST37-S1, GFP positive subpopulation of ST37; ST 37-S2M, medium fluorescent subpopulation of ST37-S1; ST37-S2H, high fluorescent subpopulation of ST37-S1; ST37-2M.12 and ST37-2M.19, clones derived from ST37-S2M; ST37-2H.43, clone derived from ST37-S2H; GFP-$S1^+$ and GFP-$S1^-$, GFP positive and negative subpopulations of a bacteriofection employing the GFP-encoding plasmid pERL3-CMVGFP-neo; GFP-$S2^+$ and GFP-$S2^-$, positive and negative subpopulations of a bacteriofection employing the GFP-encoding plasmid pERL3-EPI1, a supposedly episomally replicating eukaryotic expression plasmid [128]. Results shown are representative for at least 3 independent experiments.

To confirm these results, cells bacteriofected with plasmids other than the GFP-CFTR encoding plasmid were also examined. Again CHO-K1 cells stably transfected by *L. monocytogenes* with the GFP-expressing plasmids pERL3-CMVGFP-neo or pERL3-EPI1 showed specific bands for all 7 primer pairs (data not shown). In contrast, the neomycin resistant but GFP negative subpopulation of the pERL3-CMVGFP-neo bacteriofection (Fig. 3.8, GFP-S1$^-$) displayed a single amplification product, whereas the GFP positive fraction exhibited two such products (Fig. 3.8 GFP-S1$^+$). Cells bacteriofected with the plasmid pERL3-EPI1 were also sorted for GFP positive (GFP-S2$^+$) and negative (GFP-S2$^-$) populations and showed either no or two specific amplification products, respectively. PCR from 5 clones from a transfection using the β-galactosidase encoding plasmid pERL3-CMVβ-neo did not result in any primer specific bands (data not shown). Thus, the more homogenous a bacteriofected cell population becomes, the less DNA from the bacterial chromosome can be detected. These results indicate that integration of *L. monocytogenes* chromosomal DNA into the host cell genome occurs, but appears to be a rare event and probably involves only small fragments of bacterial DNA.

3.2 *L. monocytogenes*-mediated gene transfer *in vivo*

The original protocol of *L. monocytogenes*-mediated gene transfer was aimed at gene therapy of Cystic Fibrosis. In that case, the target cell for therapy should be accessible to the bacterium and the ability of *L. monocytogenes* to spread from cell to cell should target also stem cells of the airway epithelium. However, in this work, the potential of *L. monocytogenes*-mediated gene transfer should be explored in other applications. Recently, several species of intracellular bacteria have been shown to specifically accumulate in tumour tissues when systemically administered to tumour bearing mice [7; 71; 137; 186]. Since the original experiments were performed in immunocompromised animals, it was decided to show a proof of principle by characterizing the infection via the intratumoural route, using BALB/c mice transplanted subcutaneously with the TS/A tumour as a model system. Also, the impact of antibiotic treatment of mice on the course of infection and gene transfer should be addressed.

3.2.1 Characterization of *L. monocytogenes* infection and gene transfer in tumour bearing BALB/c mice

BALB/c mice were subcutaneously injected with tumour cells derived from the methylcholantren induced breast cancer TS/A. When tumours had reached 5 – 8 mm in diameter, *L. monocytogenes* EGD carrying the eukaryotic expression plasmid pERL3-CMVGFP were injected directly into the tumour tissue and the number of viable bacteria was established by plating dilutions of tumour and organ homogenates at different time points after the infection. Mice were divided into two groups of which one received treatment with ampicillin with the intention to control the infection and at the same time to initiate lysis of bacteria and gene transfer. The other group did not receive antibiotic treatment. As shown in Fig. 3.9, high numbers of bacteria could be found in tumours at 12 hrs post infection (p.i.). However, the bacteria were not completely contained within the tumour tissue, as could be expected from recent data [186]. Listeria were detected in liver and spleen as early as 12 hrs p.i., although at low numbers. In mice that received no antibiotic treatment (Fig. 3.9 A), Listeria were able to replicate in liver and spleen and by 60 hrs p.i., the total numbers of CFUs in these organs were comparable to those found in the tumours at the same time point. In contrast, the number of viable bacteria in the tumour tissue was constant or increased only slightly over time.

When mice were treated with ampicillin (Fig. 3.9 B), the bacterial load in liver and spleen still increased as seen at 36 hrs p.i. could be observed. The number of bacteria then was decreased at 60 hrs p.i. In the tumours, bacterial numbers decreased with time leading to a 10 to 100 fold reduction at 60 hrs p.i. Thus, despite antibiotic treatment starting at 12 hrs p.i. significant numbers of viable bacteria could still be found at later time points, indicating that efficient antibiotics treatment *in vivo* might require higher ampicillin concentrations.

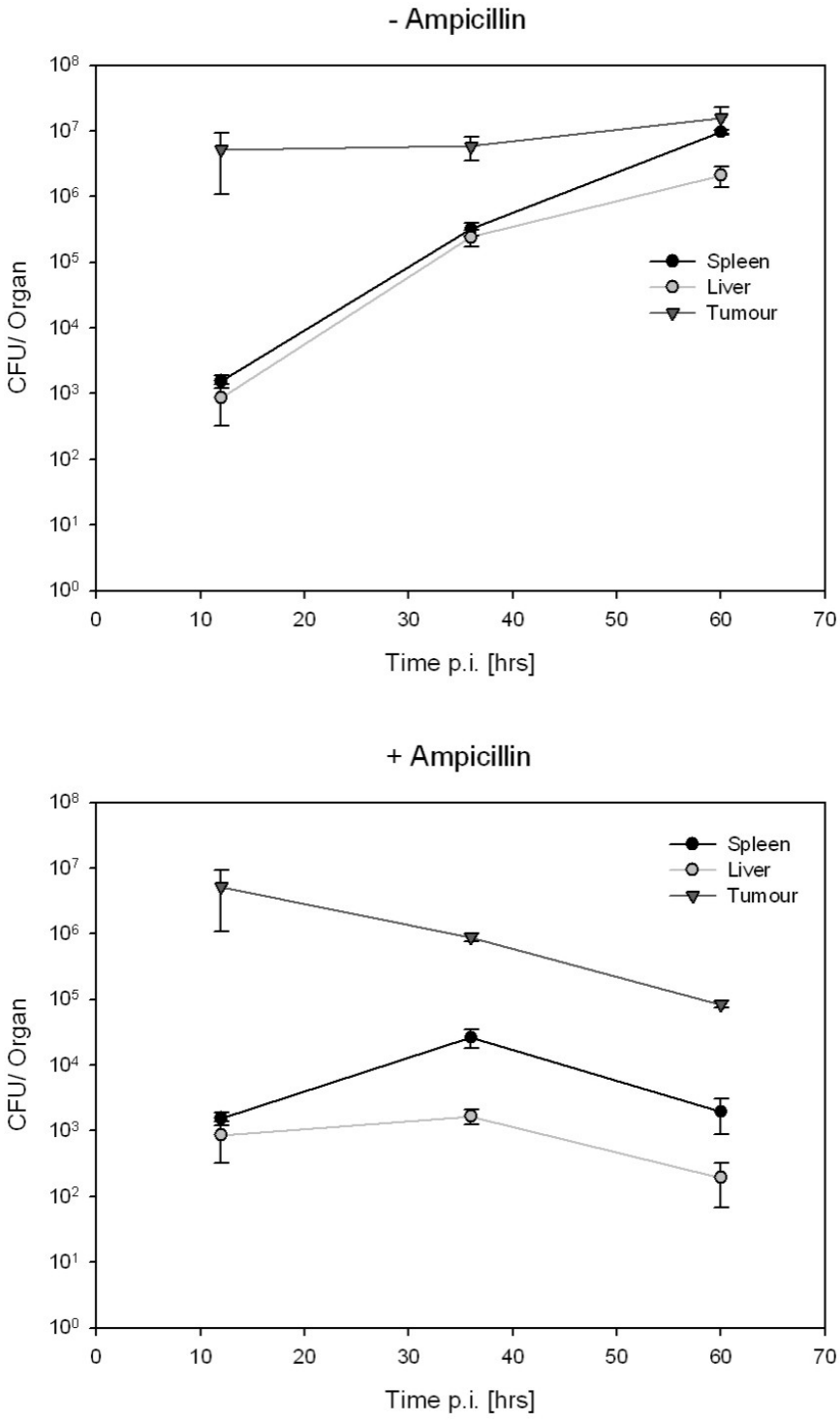

Fig. 3.9: Distribution of *L. monocytogenes* after intratumoural infection of BALB/c mice. Mice were infected with $5x10^5$ *L. monocytogenes* W491A carrying the expression plasmid pERL3-CMVGFP. Bacteria were grown in minimal medium prior to infection. Antibiotic treatment was initiated at 12 hrs p.i. Mice received 2 mg ampicillin i.p. every 12 hrs until the end of the experiment. Two individual mice per group were used. Symbols represent the average value with error bars indicating the range of the lowest and the highest values, respectively.

To see if gene transfer and transgene expression from the eukaryotic expression plasmid carried by the Listeria took place in the tumour, cells were isolated from these solid tumours and analyzed for GFP expression by flow cytometry (Fig. 3.10). Due to the isolation procedure, a large number of dead cells were observed during analysis of all tumours. However, GFP expression could not be detected in individual tumours. No difference to the non-infected controls could be observed. However, a strong influx of GR-1 positive cells, most likely neutrophilic granulocytes, into the tumour tissue was observed (Fig. 3.11). This was in accordance with the role of granulocytes in *L. monocytogenes* infection (see also Introduction).

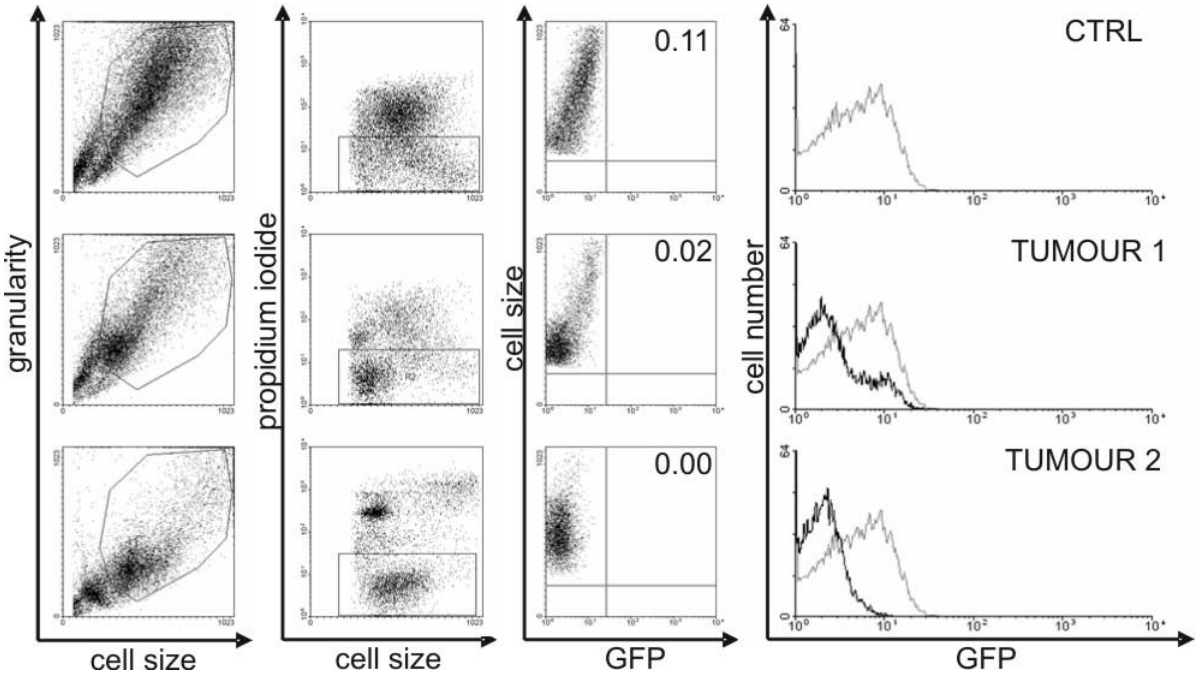

Fig. 3.10: Analysis of transgene expression after in vivo bacteriofection. Tumour cells were isolated from BALB/c mice that were infected intratumourally with $5x10^5$ *L. monocytogenes* W491A carrying the plasmid pERL3-CMVGFP. Transgene expression was analyzed 48 hrs after start of antibiotic treatment. No GFP expression could be detected in two individual tumours (tumour1 and tumour2, respectively; black lines). CTRL: tumour cells isolated from a non-infected mouse (grey lines). Numbers in the upper right quadrant represent the percentage of viable, GFP positive cells. This experiment was repeated twice with similar results.

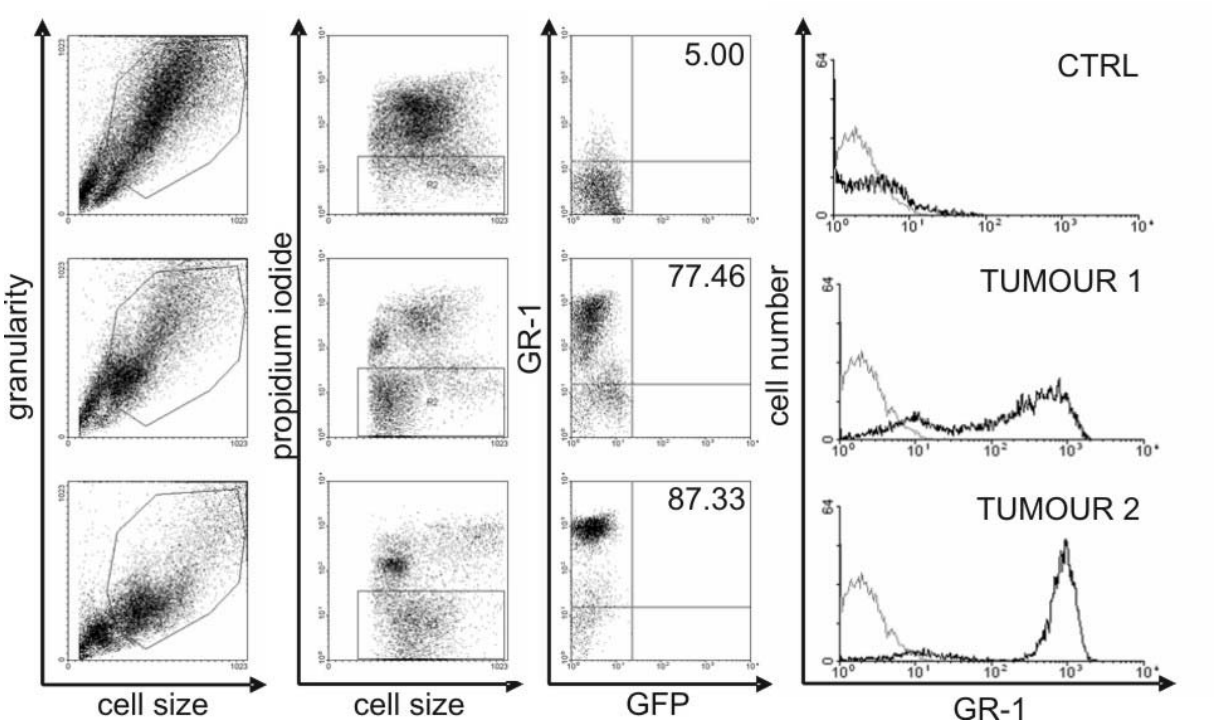

Fig. 3.11: A strong influx of GR-1 positive cells is found in tumours of mice infected with *L. monocytogenes*. Cells were isolated from tumours of BALB/c mice 48 hrs after intratumoural infection with *L. monocytogenes* W491A carrying the plasmid pERL3-CMVGFP. GR-1 expression was analyzed by using a monoclonal anti-GR-1 antibody. CTRL: cells isolated from non-infected mice. Tumour1 and Tumour2: cells isolated from tumours of two individual mice. Grey lines: cells incubated with only the secondary antibody. Black lines: cells stained with anti-GR-1 antibody. Numbers represent the percentage of viable, GR-1 positive cells.

3.3 *S. flexneri*-mediated gene transfer

Since *in vivo* gene transfer into tumour cells could not detectably be mediated by *L. monocytogenes* under our conditions, it was decided to turn the attention to another intracellular bacterium, the Gram-negative bacterium *Shigella flexneri*. Two different attenuated strains (*S. flexneri* ΔaroD and *S. flexneri* Δdap) were used to characterize infection and gene transfer first *in vitro* in different cell lines, and subsequently *in vivo* in a mouse tumour model. Growth of both strains is dependent on metabolites that are not available inside the mammalian host cell. Therefore, a treatment with antibiotics to kill intracellular bacteria and initiate gene transfer should not be necessary.

3.3.1 Characterization of *S. flexneri*-mediated gene transfer in different cell lines *in vitro*

S. flexneri ΔaroD and *S. flexneri* Δdap, both carrying the eukaryotic expression plasmid pCMVGFP were used to bacteriofect three different cell lines (BHK-21, CT26 and HEp-2) *in vitro*. As shown in Fig. 3.12, up to 2.6% of HEp-2 cells bacteriofected with the ΔaroD strain at a MOI of 78 expressed GFP 24 hrs after bacteriofection. For BHK-21 and CT26 cells, approximately 1.5% of the cells were transfected. Transfection rates were not significantly enhanced when higher MOIs of up to 500 were used (data not shown). Furthermore, transfection rates were comparable with and without antibiotic treatment, demonstrating that death of intracellular bacteria and gene transfer does not require antibiotics.

Similar results were obtained when using the Δdap strain of *S. flexneri* at a MOI of 300, although the overall transfection rates were slightly lower compared to the ΔaroD strain. Small differences were also seen between cells treated with antibiotics and non treated cells, with lower transfection rates in non treated HEp-2 and BHK-21 cells, while in CT26 cells, higher transfection rates were observed in non treated cells. However, this could be due to variations within the experiments.

GFP has proved very useful as a reporter gene to characterize bacteriofection *in vitr*o, especially because it allows the detection of single bacteriofected cells and the level of expression per cell by flow cytometry. Nevertheless, it is not a good reporter gene for *in vivo* studies in our case. Cells that are infected with bacteria (and therefore are dying or about to die) show high levels of autofluorescence that cannot be easily distinguished from low levels of GFP expression. Therefore fire fly luciferase was used as an alternative reporter gene for Shigella-mediated gene transfer. This enzyme requires less time to become detectable compared to GFP. In addition, extremely low background activity is observed in mammalian cells and tissues. Therefore, also low expression levels can be detected by measuring luminescence.

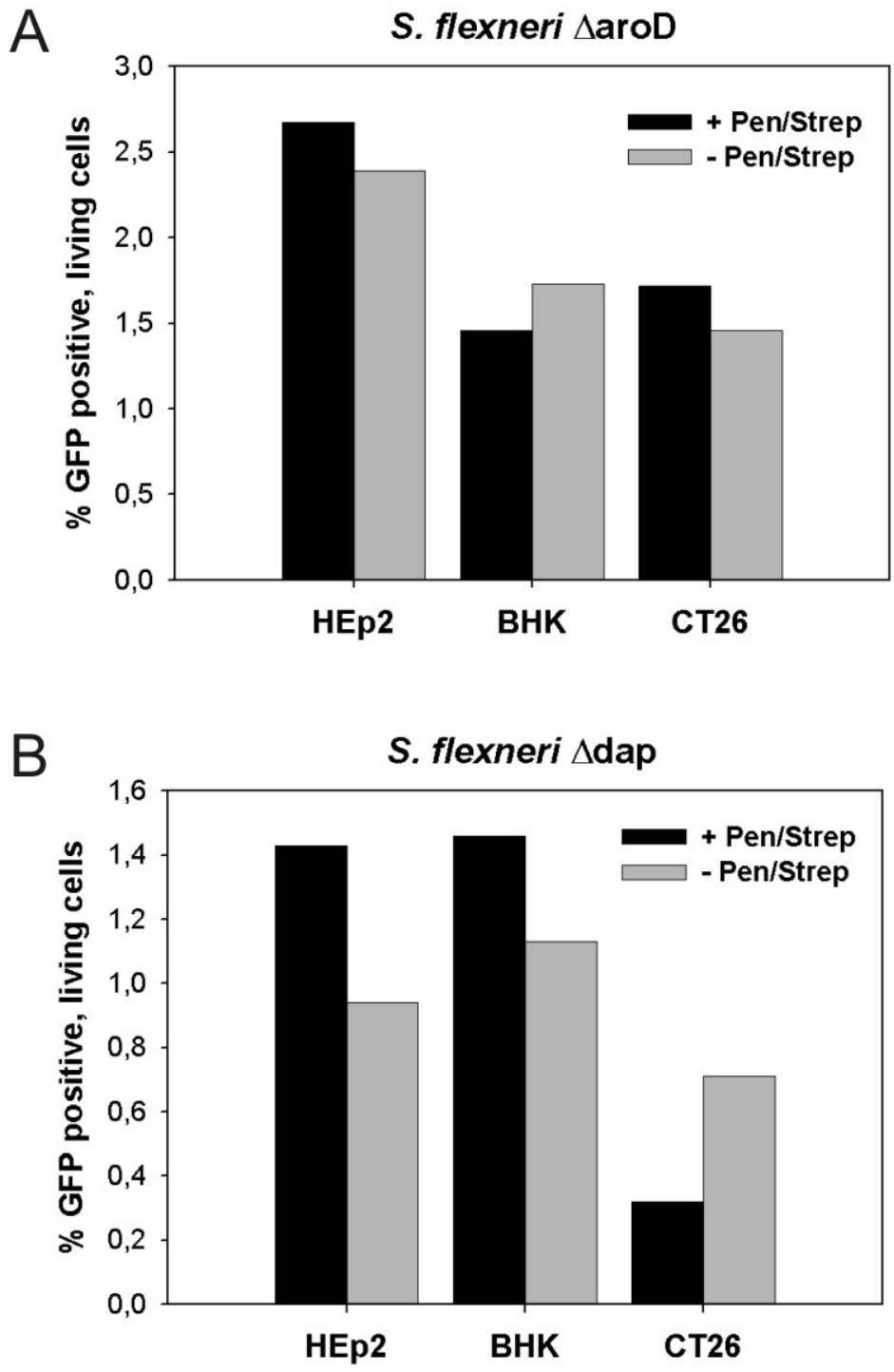

Fig. 3.12: *In vitro* bacteriofection of different cell lines with attenuated *S. flexneri* strains. GFP expression was analyzed 48 hrs after bacteriofection (**A**) HEp-2, BHK-21 and CT26 cells were bacteriofected with *S. flexneri* ΔaroD carrying the expression plasmid pCMVGFP at a MOI of 78. (**B**) HEp-2, BHK-21 and CT26 cells were bacteriofected with *S. flexneri* Δdap carrying the expression plasmid pCMVGFP at a MOI of 300. + Pen/Strep: penicillin/ streptomycin was added to the cells 4 hrs p.i. – Pen/Strep: no penicillin/ streptomycin was added to kill intracellular bacteria. The percentage of viable, GFP positive cells is shown as determined by flow cytometry.

To test the Shigella ΔaroD and Δdap strains carrying the eukaryotic expression plasmid pCMV-luc-m2A (Bauer, H.; unpublished) for their *in vitro* transfection efficiency, again the three cell lines HEp-2, BHK-21 and CT26 were used. After 24 hrs and 48 hrs, the number of intracellular bacteria was determined in parallel to luciferase activity in the cell lysates (Fig. 3.13). After infection with *S. flexneri* ΔaroD, luciferase activity was detected in all cell lines tested, although considerable differences were observed between the three cell lines (Fig. 3.13 A). The highest activity after 24 hrs was detected in BHK-21 cells compared to HEp-2 and CT26 cells, which showed comparable activities. Luciferase expression had increased in BHK-21 and HEp-2 cells, and reached comparably high levels in these two cell lines by 48 hrs, while expression in CT26 cells remained constantly low. At this time, only very few viable intracellular bacteria could still be found (Fig. 3.13 C). This again indicates that antibiotic treatment is not required when using the attenuated *S. flexneri* ΔaroD strain.

The luciferase activity in all cell lines was lower when the *S. flexneri* Δdap strain was used for bacteriofection (3.13 B). At 24 hrs, activity was again highest in BHK-21 cells, while in CT26 cells, expression barely raised over the background luminescence. At 48 hrs, the expression level increased in all cell lines, again most strongly in BHK-21 cells. For unknown reasons, the number of remaining viable, intracellular bacteria did not correlate with the level of expression (Fig. 3.13 D). In most cases, numbers of viable Δdap bacteria were higher compared to ΔaroD bacteria.

3.4 *S. flexneri*-mediated gene transfer *in vivo*

To investigate whether the two Shigella strains tested *in vitro* are suitable for gene transfer into tumour cells *in vivo*, a similar model system was used as described above for Listeria-mediated gene transfer. Two modifications were introduced: first, the tumour cell line CT26, which is derived from a mouse colon carcinoma, was used instead of TS/A, and second, GFP was exchanged for luciferase as a reporter gene for the reasons mentioned above. In addition, three different routes of infection (intraperitoneal, intravenously and intratumoural) were employed to test the ability of *S. flexneri* to accumulate in tumour tissues when administered to BALB/c mice.

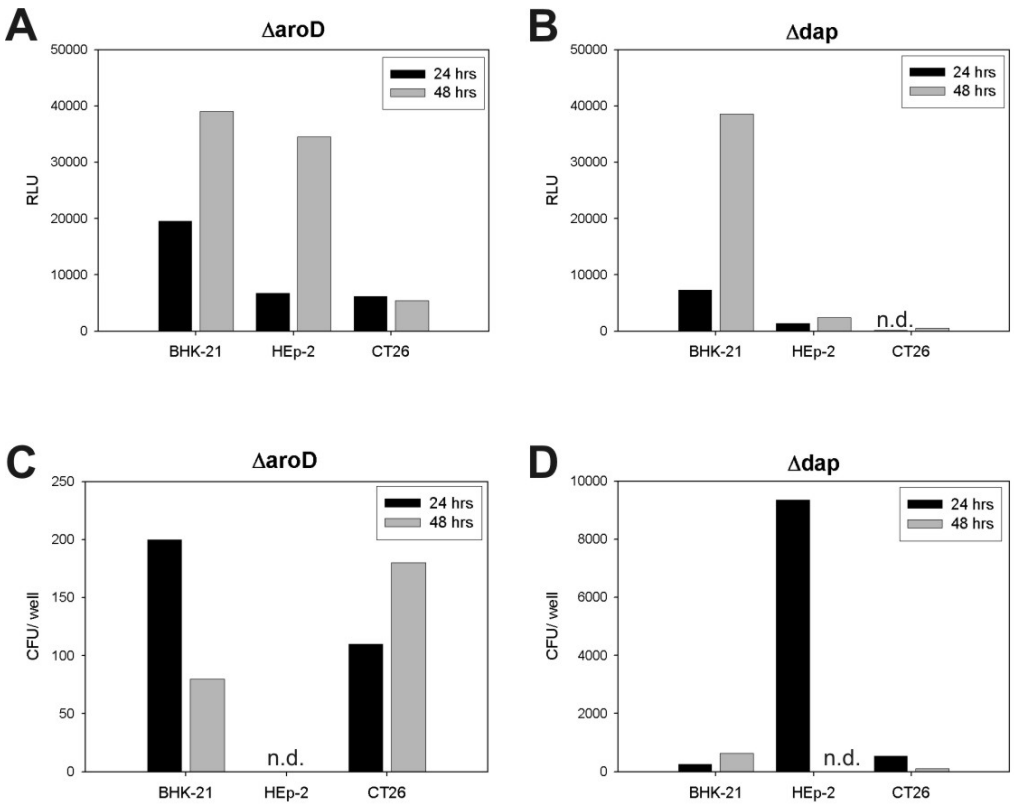

Fig. 3.13: *In vitro* bacteriofection of different cell lines with attenuated *S. flexneri* strains carrying the expression plasmid pCMV-luc-m2A. Luciferase expression and the number of viable, intracellular bacteria were determined 24 and 48 hrs after bacteriofection. In (**A**) and (**B**), BHK-21, HEp-2, and CT26 cells were bacteriofected with *S. flexneri* ΔaroD and Δdap, respectively and enzymatic activity of luciferase was determined. (**C**) and (**D**) show the number of intracellular ΔaroD and Δdap bacteria, respectively, as determined by plating on TSB plates with ampicillin. n.d.: not detectable

3.4.1 Characterization of *S. flexneri* infection and gene transfer in tumour bearing BALB/c mice

After infection with the two *S. flexneri* strains ΔaroD and Δdap, the number of viable bacteria in liver, spleen and tumour tissue was determined by plating serial dilutions of tumour and organ homogenates at different time points after infection. When the bacteria were given by the intratumoural route, a distribution pattern of bacteria was found similar to intratumoural infection by *L. monocytogenes* (Fig. 3.14 A). Using the ΔaroD strain, high numbers of Shigella were detected in the tumours of mice 24 hrs after infection, and the number of viable bacteria stayed constant during 48 and 72 hrs. This is in contrast to the observations made by the *in vitro* experiments, where only very few bacteria were found at these late time points p.i.

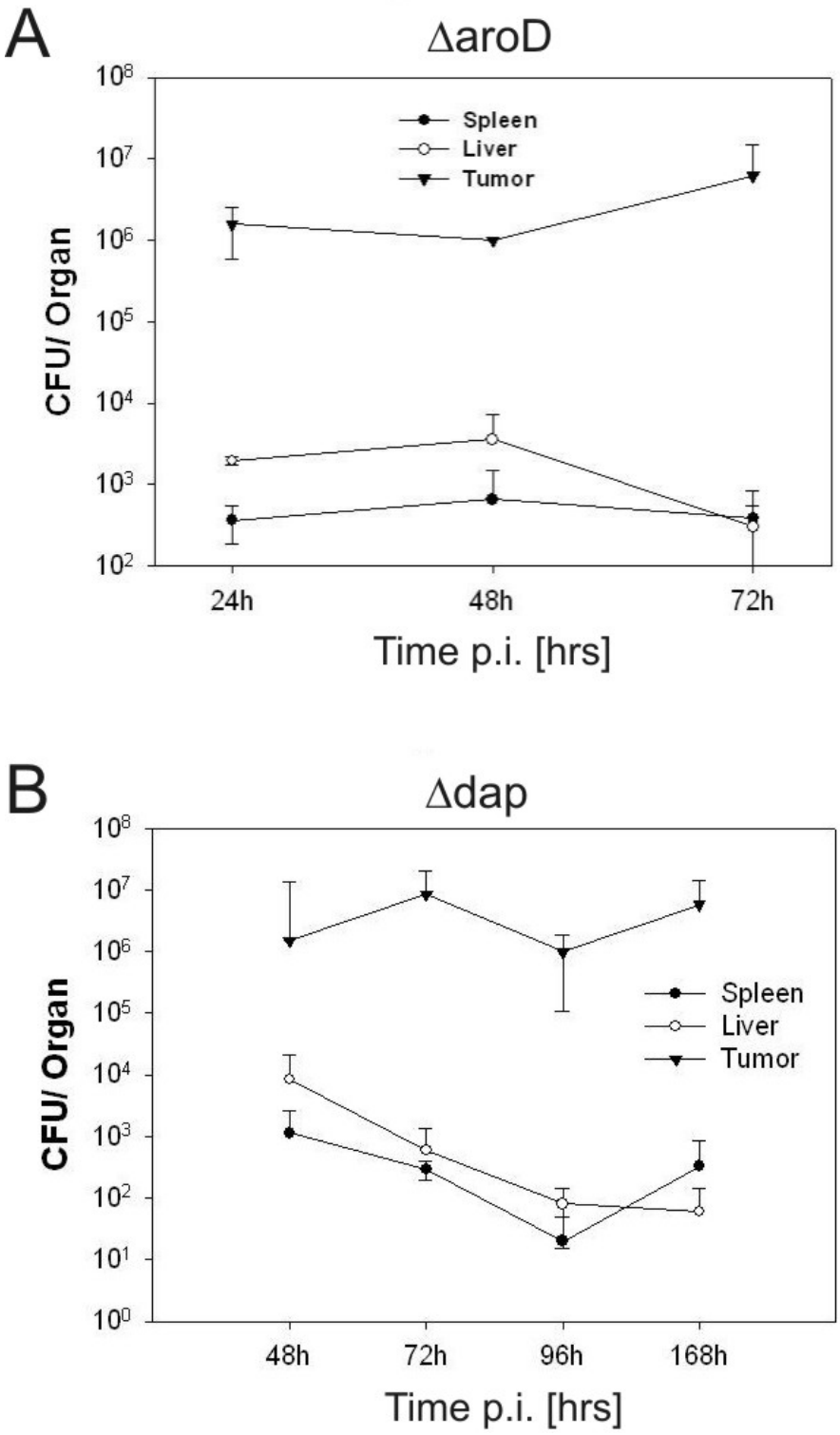

Fig. 3.14: Distribution of attenuated *S. flexneri* strains in tumour-bearing BALB/c mice after intratumoural infection. Mice were infected with 10^6 *S. flexneri* ΔaroD (**A**) or 10^8 *S. flexneri* Δdap (**B**). The number of viable bacteria was determined by plating of homogenates of tumours and organs at indicated time points. Values represent the average of three individual mice per group (mean +/- s.d.).

Bacteria could also be detected in the liver and spleen of mice for a period of 72 hrs, albeit at much lower numbers than in the tumour. In spleen, the bacterial load stayed constant during the observation period, while in the liver it decreased.

After systemic administration via the intraperitoneal or intravenous route, no bacteria were detected at any time point in any organ or tissue (data not shown). This is in accordance with the fact that *S. flexneri* is not infectious in mice.

Alternatively, mice were infected with Shigella ∆dap intratumourally (Fig. 3.14 B) and the bacterial load in tumour, liver and spleen was monitored over a period of 7 days. Again, high numbers of viable bacteria were found in tumours until day 4. On day 7, a decrease of the bacterial burden in the tumour was observed in a single mouse, while it remained high in the others. CFUs were significantly lower in liver and spleen, and the bacterial load decreased slightly with time. When Shigella ∆dap was given by the intraperitoneal or intravenous route, only very few bacteria were detected in tumours and the organs tested (data not shown), demonstrating that *S. flexneri* ∆dap as well as ∆aroD do not accumulate specifically in tumour tissues in BALB/c mice after systemic administration.

Since Shigella-mediated gene transfer was observed *in vitro*, the two recombinant attenuated strains were also tested for their ability to mediate gene transfer *in vivo*. CT26 bearing BALB/c mice were infected intratumourally with *S. flexneri* ∆aroD and ∆dap carrying the eukaryotic expression plasmid pCMV-luc-m2A. Transgene expression was monitored at 24 and 48 hrs after infection (Fig. 3.15 A). At the same time, the number of viable bacteria in the tumour tissue was established by plating (Fig. 3.15 B).

Since the CMV immediate early promoter shows some activity also in bacteria [52], it was necessary to establish that the detected luciferase activity was not derived from bacterial expression but resulted from *in vivo* gene transfer. To account for this possibility, an aliquot of the original inocculum was lysed under the same conditions as the tumour tissue and the supernatant was analyzed for luciferase.

Luciferase activity was detected in tumours of infected mice, although the signal strength varied considerably between individual animals (Fig. 3.15 A). However, the bacteria also produced some luciferase, despite the eukaryotic CMV promoter used to drive luciferase expression (data not shown). Additionally, the signal intensity correlated with the number of viable bacteria in tumours. Elevated levels of luciferase activity were observed in tumours, but at the moment, it cannot unambiguously be

concluded that gene transfer took place and the luciferase detected was synthesized by the eukaryotic cells. Since *in vivo* gene transfer with *S. flexneri* was demonstrated in other systems [147; 154], it can be assumed that gene transfer can take place to a certain extend also under the conditions found here. Thus, further work is required to establish bacteria-mediated gene transfer *in vivo* for gene therapy of cancer.

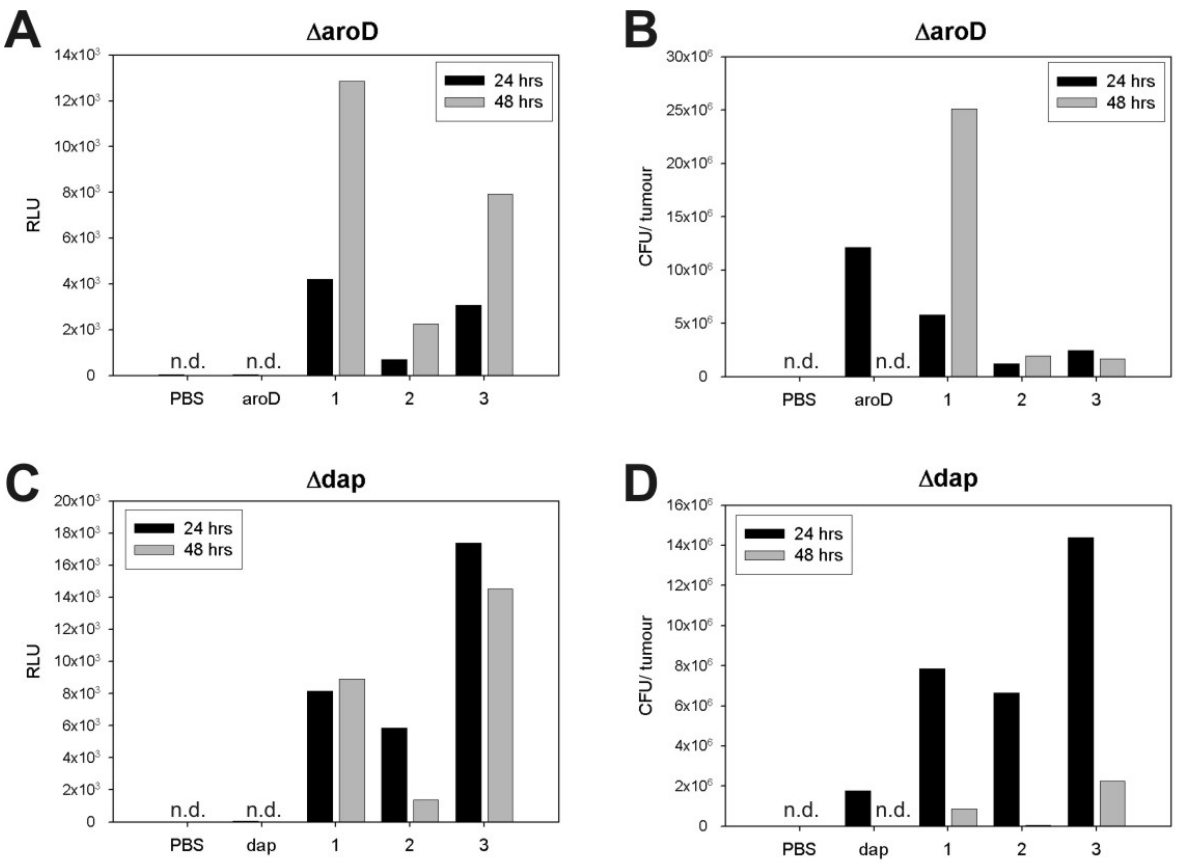

Fig. 3.15: *In vivo* gene transfer and number of viable bacteria in tumour tissues after intratumoural infection of CT26 bearing BALB/c mice with $5x10^7$ *S. flexneri* attenuated strains. (**A**) and (**C**): Luciferase activity 24 and 48 hrs p.i. in tumour tissues of mice infected with *S. flexneri* ΔaroD and Δdap, respectively, carrying the plasmid pCMV-luc-m2A. (**B**) and (**D**): Numbers of viable bacteria in tumour tissues were established by plating of tumour homogenates 24 and 48 hrs p.i. PBS: Mice were injected with PBS; aroD, dap: mice were infected with the parental bacterial strain without a plasmid. 1, 2 and 3 are individual mice. n.d.: not detectable.

4 Discussion

4.1 The mechanism of DNA transfer by *L. monocytogenes*

An *in vivo* application of the Listeria-mediated DNA transfer system, for instance to treat monogenic disease or cancer, has to satisfy several prerequisites, the most important one being a sufficient transfection rate of the target tissue. At the moment, the number of cells transfected even *in vitro* is still rather low. It is therefore of utmost importance to find out the mechanism of DNA transfer in molecular terms, in order to understand its limitations and then, subsequently, eliminate such restrictions.
There are three obvious steps that might limit the process of bacteria-mediated gene transfer: I) invasion of the host cell; II) bacterial lysis and plasmid release; III) stability and transport of the expression plasmid to the nucleus.

Invasion of the host cell
From our former data [60; 88] and data presented here it seems that invasion of the host cell by the bacteria is not a critical step since most cells are infected and contain several bacteria. When *L. monocytogenes* were grown in minimal medium which leads to the upregulation of virulence factors involved in cell invasion, a 5- to 6-fold increase in the numbers of intracellular bacteria during infection was observed. However, only a 2- to 3-fold increase of transfected cells could be detected. Thus, augmented invasion of the host cell can enhance transfection efficiencies, but transfer efficiency is not directly correlated with invasiveness of the bacteria. This leads to the conclusion that only a fraction of the intracellular bacteria are able to eventually transfer their plasmid load to the nucleus of the host cell.

Bacterial lysis and plasmid release
The idea that only very few bacteria transfer their plasmid was further substantiated in an experiment in which cells were cotransfected with two different plasmids by *L. monocytogenes*. Relatively high numbers of cells were found that expressed only one transgene, suggesting that normally only a few or even a single plasmid is transferred to the nucleus. However, the high transgene expression levels that were observed in transfected cells are opposed to the suggestion that the transgene is

expressed from only a single copy of the plasmid. Thus, the transfer of bundled plasmids derived from a single bacterium under these conditions is more likely.
Bacteria are efficiently lysed within the cytosol of the host cell upon antibiotic treatment. Their cell walls were disrupted quickly and the bacterial content was released as revealed by electron microscopy. This was confirmed by showing that the GFP protein expressed by the bacteria is quickly distributed within the cytosol of the eukaryotic host cell upon bacterial lysis. Virtually all cells that were initially infected contained evenly distributed GFP 48h after antibiotic treatment. Consequently, the efficiency of bacterial lysis and release of the bacterial content into the cytosol of host cells should be high enough to allow effective gene transfer.

Stability and transport of the expression plasmid to the nucleus
One possibility why transfection rates are low could be that the DNA transferred by bacteria is degraded in the host cell's cytoplasm. It could be shown, however, that low transfer rates are not due to rapid degradation of the plasmids since large amounts of intact plasmids could be recovered even 48 h after initiation of antibiotics treatment. Rather, the plasmids appear to be associated with macromolecules or organelles as indicated by their sedimentation properties. Spiking the lysates with control plasmids showed that the high sedimentation rate of the bacterially introduced plasmids is most likely not an artefact of the lysis procedure. Most of the control plasmids could be recovered in the slowly sedimenting fraction containing the cytosolic proteins during these experiments.
This was corroborated by an experiment using a plasmid from which the transgene should be expressed in the cytosol of the host cell. After transfer by *L. monocytogenes*, the transgene was not expressed. This again suggests a strong association of the plasmid with cellular or bacterial components and would also explain the discrepancy found between plasmids introduced by calcium phosphate transfection as compared to bacteriofection.
Thus, the most important limitation for an efficient gene transfer using *L. monocytogenes* as carrier, and its application *in vivo*, is the association of the plasmid DNA with macromolecules or organelles after the lysis of the bacteria.
The next step should be now to further characterize these complexes. For instance, the fractionation should be carried out in a more sophisticated way to determine the exact size of the complexes. If organelles are involved, they should be characterized

by microscopical and biochemical means. Upon these results, it might be possible to modify the plasmids or the associated macromolecules in a way that facilitates transfer of DNA to the nucleus. For instance, DNA binding proteins might be expressed in the bacteria that contain eukaryotic nuclear localisation signals which might solve the present problems.

4.2 Integration of bacterial genomic DNA into the host cell genome

Since high bacterial MOIs were used under our bacteriofection conditions, it was plausible that fragments of the bacterial chromosome can also reach the nucleus of the host cell. It was therefore important to clarify the fate of the bacterial genomic DNA released from lysed *L. monocytogenes* in the host cell cytosol. To avoid listerial DNA that might have been only transiently present after infection, we employed stable bacteriofectants that had been in culture for a considerable time. Under these circumstances, bacterial DNA is diluted out due to cell division unless it is integrated into the genome of the host cell. Only then can it be replicated and stably distributed to the daughter cells.

Chromosomal DNA of *L. monocytogenes* was detected in the genomic DNA of the transfected host cell. However, the more homogenous a cell population was, the less bacterial DNA was detected. In sorted subpopulations only a few specific signals could be observed, while in all of the clones analyzed so far, no bacterial DNA was found.

The sequences that are covered by the primer pairs represent only about 0.15% of the *L. monocytogenes* chromosome. Thus, bacterial DNA could have been present but was not detected. Despite of this possibility, one can conclude that integration of bacterial chromosomal fragments, although clearly taking place, is a rare event under our conditions. Whether the mechanisms leading to plasmid and bacterial DNA transfer are identical remains to be investigated.

Integration of bacterial chromosomal DNA could represent a severe safety risk.

Integration into the LMO2 proto-oncogene locus by the retroviral vector used for the gene therapy of an immunological defect in two children has recently resulted in the induction of cancer [56]. This was most likely due to a strong retroviral enhancer activity on the LMO2 gene promoter. It is however unlikely that a similar scenario

takes place due to the integration of bacterial DNA. Bacterial control elements are notoriously weak in the mammalian cells if active at all.
Integration of DNA fragments could also result in the inactivation of a tumour suppressor gene. However, this will take place only on one chromosome leaving the second allele intact. Nevertheless, despite the low frequency that was suggested by the experiments described, a finite risk remains and the most effective solution of this problem would be to significantly raise the efficiency of the gene transfer. This would allow a dramatic reduction of the MOI and consequently lead to a strongly reduced risk of cotransfer of bacterial chromosomal DNA.

4.3 Bacteria-mediated gene transfer in tumour therapy

4.3.1 *L. monocytogenes*

So far data on successful transfer of expression plasmids using *L. monocytogenes* as carrier are rather limited. In fact, only low transfer rates were reported into peritoneal macrophages in the cotton rat. Therefore, it was argued in the present work that if Listeria of the current design can be used as vectors, direct injection into tumour tissue should be the most promising approach. The target cells should be directly infected and cell to cell spread should help to infect large areas of the tumour. In addition, it was important to determine whether antibiotic treatment also reaches the bacteria in the tumour and whether the infective bacteria can still be controlled after the initial phase of growth at this site.
High numbers of bacteria were observed in tumour tissues over a time period of 60 hrs in mice without antibiotic treatment but were not contained in this tissue. Bacteria disseminated to the liver and the spleen of mice, where they replicated rapidly. Two possibilities could explain this phenomenon. The tumour tissue could serve as a kind of depot and bacteria might be transported continuously via blood or lymph to other organs. Alternatively, it is possible that the bacteria enter the bloodstream through injuries inflicted to the tumour blood vessels during injection.
Application of ampicillin resulted in a decrease of the bacterial burden in all tissues investigated, although significant numbers of bacteria could still be found. This might be due to a time period of treatment too short to be effective. On the other hand,

some areas of the tumour might not be well vascularized, leading to poor delivery of the antibiotic and protection of the residing bacteria.

Reporter gene expression after *L. monocytogenes* mediated gene transfer was not observed under the conditions employed. Several reasons could account for this finding. First of all, from the data presented here, it is not evident that the Listeria found in the tumour tissue are located inside the tumour cells. However, intracellular location is absolutely essential for efficient gene transfer to occur [60]. This matter should be addressed by using immunehistochemistry of infected tumour tissue. Furthermore, as discussed above, obviously only a fraction of all bacteria transfer their plasmid to the nucleus of the host cell. Higher transfection rates should lead to detectable transfer also *in vivo*. An alternative explanation could be that the innate immune system removes cells that were invaded by *L. monocytogenes*. High numbers of neutrophilic granulocytes were found in tumour tissues after infection. It is therefore conceivable that infected and potentially transfected cells are killed as has been shown for infected hepatocytes [19]. Studies in which neutrophils are depleted before infection, e.g. by the use of monoclonal antibodies, should clarify this issue.

Using GFP as a reporter gene *in vivo* might also have added to the lack of detection of *in vivo* transfected cells. Autofluorescence is high in necrotic tissue. Therefore cells that express low levels of GFP protein might have escaped the detection because they do not exceed the background fluorescence. Choosing an alternative reporter that displays a low background activity, e.g. luciferase as used for Shigella, should solve this problem.

4.3.2 *S. flexneri*

As alternative carrier, *Shigella flexneri* was employed, a bacterium for which *in vivo* gene transfer has been shown before [146; 154]. First, proof of principle was required that this bacterium transfers plasmids into the tumour cells that were used in the model system. In addition, it was necessary to compare the transfection efficiency when lysis was exclusively dependent on the attenuation or when antibiotics were added. Three different cell lines were bacteriofected *in vitro* by either *S. flexneri* ΔaroD or *S. flexneri* Δdap. First, GFP was used as reporter gene to quantify the number of transfected cells and to analyse expression levels.

Using the ΔaroD strain, gene transfer to HEp-2, BHK-21 and CT26 cells could be demonstrated and differences between transfections with or without antibiotic treatment were marginal. Apparently, this strain lyses efficiently in the host cell due to its inability to synthesize aromatic amino acids. The Δdap strain is also able to mediate gene transfer into the three cell lines, but is less efficient than the ΔaroD strain. Again, no significant differences were observed with or without antibiotics. Thus the metabolic attenuation is sufficient to ensure lysis of the bacteria in the host cell.

In the studies by Krusch et al. [88] and in the present work it could be established that disruption of the bacterial cell wall is important for efficient gene transfer. Therefore, the lower transfection rates mediated by the Δdap strain compared to the ΔaroD strain were unexpected, since the mutation in the cell wall synthesis pathway should lead to especially efficient lysis of the bacteria and disruption of the cell wall. However, for the time being it can not be excluded that the differences in transfer efficiency are due to the diverse genetic background. The two bacteria were derived from different parental strains which might influence several steps required for gene transfer. In agreement with this was that the Δdap strain was less invasive than the ΔaroD strain. Grossly, similar results were obtained when luciferase was tested as reporter.

For the *in vivo* transfer of expression plasmids into tumour tissue, *Shigella flexneri* was first tested for enrichment in tumour tissue after i.v. and i.p. administration. As mentioned before, accumulation in tumours has been described for several bacterial species. However, no bacteria could be found at any time point in the tissues tested. Since *Shigella flexneri* is not pathogenic in mice, this was not unexpected. Most likely, the innate immune system eliminates the bacterium almost immediately after administration. This does not necessarily exclude that Shigella can have tumour targeting properties in its natural hosts. Therefore, these experiments should also be performed in animals that are susceptible for Shigella infection.

In contrast, direct intratumoural application revealed interesting results. Bacteria could survive for an extended period of time, actually much longer than would be expected from their behaviour in the *in vitro* experiments. In addition, bacteria were found in spleen and liver under these conditions. This seems to be in contrast with the results obtained by i.v. or i.p. injection. Most likely the tumour serves as immune-

privileged reservoir that is rich in nutrients and from which bacteria can disseminate into other tissues.

In vivo gene transfer was tested using luciferase as reporter. Here, enzymatic activity could be detected in tumour lysates. However, the CMV promoter used for expression is also active to a certain degree in bacteria. Therefore, the evidence for gene transfer can be taken as suggestive but not as compelling. This problem must be solved by either taking a promoter that is completely silent in the bacterial carrier or by constructing a reporter that can only be active in the host cell. This could be achieved for instance by introducing an intron into the reporter gene or by employing a reporter whose activity relies on glycosylation.

The above results call into question the genuineness of the results obtained after *in vitro* bacteriofection using luciferase as reporter. However, in this case, the number of viable intracellular bacteria generally decreased from 24 to 48 hrs, while the luciferase signal strength increased at the same time points. Taking into account the short biological half life of the luciferase protein, which is approximately 2 hrs [63], it is sure that the detected luciferase activity was expressed from a plasmid in the host cell nucleus after transfer.

Taken together, from this work it is apparent that intracellular bacteria as gene shuttles are still at the initial phase of development. Nevertheless, they have attracted already substantial attention. Bacteria as carriers for mucosal DNA vaccines are already completely accepted by the scientific community. It can be foreseen that improvement of transfection efficiencies in the future will also allow such microorganisms to be employed in gene therapy of complex diseases.

5 Summary

Several aspects of bacteria-mediated gene transfer *in vitro* and *in vivo* have been addressed in this work. First, the mechanisms involved in plasmid transfer from the intracellular bacterium *L. monocytogenes* to eukaryotic host cells were investigated.

It could be shown that the initial step during bacteriofection, namely invasion of the host cell, is not limiting for efficient gene transfer. Furthermore, bacteria are efficiently lysed upon antibiotic treatment and release their content to the cytosol. Despite of this, it became obvious that only a fraction of the intracellular bacteria transfer their plasmid load to the nucleus of the host cell, as was shown by co-transfer of two different expression plasmids by *L. monocytogenes*. Further experiments suggested that contrary to the original idea in which the plasmids are simply released upon bacterial lysis the plasmids are associated with high molecular weight components. However, not only plasmid DNA was transferred to the nucleus of the host cells, but also to a small degree bacterial chromosomal DNA was transferred and integrated in the host cell genome.

These findings suggest further studies towards the investigation of the plasmid transfer pathway and its rational improvement. Enhancing the transfer efficiencies should drastically lower the dose of bacteria required to achieve effective bacteriofection and thus render integration of bacterial DNA negligible.

In the second part of this work, *L. monocytogenes* and *S. flexneri* were tested as gene transfer vehicles for cancer therapy. The dissemination of both bacteria was analyzed after infection via the intratumoural route. Unexpectedly, the bacteria were not contained within the tumour tissue but were also found in liver and spleen of mice. Viable attenuated Shigella were detected in mice as long as 7 days p.i. However, when mice were systemically (via the intraperitoneal or intravenous route) infected with attenuated *S. flexneri*, the bacteria were rapidly cleared from the blood stream and did not accumulate in tumours or organs, as described before for other bacterial species.

Gene transfer from bacteria to tumour cells *in vivo* could not clearly be demonstrated for Listeria under the conditions used. In contrast, some implication of gene transfer by Shigella was found. Here, refinement of the technical settings should allow the definite detection of even low levels of transgene expression and permit distinction of eukaryotic and prokaryotic expression. In summary, improvements that will be

suggested by extending the present work will result in better gene transfer abilities and will reveal the great potential of such bacteria as DNA carriers.

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